Prediction of wine color from phenolic profiles of red grapes

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PREFACE

This PhD thesis concludes my Industrial PhD project, from February 1st 2005 to February 29th 2008. The project has been a collaboration between FOSS and the Technical University of Denmark under the Danish Industrial PhD framework. The work has mainly been carried out at FOSS, the Department of Chemical and Biochemical Engineering, and the Department of Systems Biology. The project was financed by FOSS and the Danish Ministry of Science, Technology and Innovation.

First I want to thank my colleagues at FOSS who have been involved in starting up the project, discussing the challenges and ideas of the project, and steering the project. My special thanks in this regards goes to my supervisor Max Egebo and people manager Dorthe Kjær Pedersen who kindly joined the project after some reorganizations in the company. Many thanks also to Björn Rechinger and Anders Bro Bendtsen for making a big effort in starting up the PhD project. Also thanks to product manager Torben Selberg for discussions on the industrial applications of the work and helping with contacts in the wine industry. Finally thanks to all colleagues in Team Greenhouse for receiving me well. I am also deeply thankful to trainee students Hans Henrik Malmborg Werge, Benoît Blachéz, and Simge Demiray for the effort and hard work you have carried out during your stays with FOSS.

Thank you to Dr. Marius Lambrechts and all the colleagues at Distell for kindly receiving me in the research group during my stay in South Africa. I am also deeply grateful to Mark van der Walt and the colleagues in Rhine-Ruhr for the kind hospitality and taking care of so many practical issues during my stay in South Africa. Special thanks also to Laboratories Dubernet and Cabinet d'Ingénieurs Conseils en Viticulture for collecting grapes for this study.

A special thanks to Professor Anne S. Meyer for being a great supervisor and mentor. Your outstanding scientific insight and never failing enthusiasm have been invaluable throughout the project. Finally thanks to all colleagues in the Center for Bioprocess Engineering for the always interesting discussions and pleasant social events.

Jacob Skibsted Jensen
Kgs. Lyngby, May 2008

SUMMARY

The phenolic composition of red wine is important for several organoleptic properties of the wine, including wine color, mouthfeel properties, bitterness, and flavor. The color of red wine is an important quality parameter of the wine, and the color intensity of red wine has been reported to correlate with wine quality grading. It is the phenolic compounds in red wine that are responsible for the wine's color properties, and obviously these phenolics to a large extent originate from the grapes. However, the relation between the phenolic composition of grapes and the phenolic composition and color attributes of red wine is complex. Furthermore, existing protocols for phenolic extraction from grapes are typically time consuming and labor intensive and thus not compatible with routine analysis at the wineries. The provision of rapid methods to obtain objective data on the phenolic composition and wine color potential of grapes could be a valuable support for important decisions in the wine industry, such as optimal harvest time, pricing of grapes, optimal processing conditions, and segregation of the grapes. On this base the primary hypotheses to be investigated in this PhD work were formulated:

- It is possible to define a robust general protocol for fast extraction of the phenolic compounds from grapes.
- It is possible to predict color attributes and phenolic compositions of red wine from the phenolic composition of grapes.
- It is possible to use mid infrared spectroscopy to measure the phenolic composition of red grapes.

The objective of this PhD project then was to investigate the prediction of wine color attributes from analysis of the phenolic composition of grapes. This included the development of a fast protocol for the extraction of grape phenols and an investigation of the feasibility of using Fourier transform mid infrared (FT-MIR) spectroscopy to analyze the phenolic composition of grapes and wines.

The influence of several factors on the extraction degree of total phenols and anthocyanins from grapes by solvent extraction was evaluated. Both extraction temperature and solvent levels of ethanol and hydrochloric acid were found to exert highly significant effects on the extraction degrees of both anthocyanins and total phenols. An optimized extraction procedure was defined, which on average allowed a high extraction degree of anthocyanins (91.5 %) and total phenols (81.8%) from grape homogenate with only 5 minutes of solvent contact. The extraction procedure was tested for eight different cultivars and concluded to give consistent results across the cultivars.

The relation between the phenolic compositions of grapes and the corresponding red wines was then investigated by use of the developed grape extraction protocol and wines produced by microvinification. The average proportion of the grape phenols recovered in the wines was low for total phenols (0.44), tannins (0.32), and anthocyanins (0.31), intermediate for (+)-catechin (0.75) and polymeric pigments (0.98) and high for gallic acid (7.9). Good direct relationships between the grape and wine phenols were observed for anthocyanins ($r = 0.93$), total phenols ($r = 0.88$), (+)-epicatechin ($r = 0.95$), and (+)-catechin ($r = 0.95$), while the direct relationships for the other phenolic classes were less evident. Using a multivariate approach to predict

the phenolic composition of wine from the detailed phenolic profile of grapes gave only minor improvements. The multivariate approach however did improve prediction of polymeric pigments, due to a strong correlation between grape anthocyanins and wine polymeric pigments ($r = 0.87$).

The detailed phenolic composition of grapes allowed prediction of several wine color attributes of pH normalized experimental wines using a multivariate approach. It was however found, that measurement of the concentration of anthocyanins in the grapes to a large extent was sufficient to predict the pH normalized wine color attributes. With residual predictive deviation (RPD) values between 2.4 and 5.7 it was possible to predict the following wine color attributes from anthocyanin measurements of the grapes: Color intensity, total wine color, wine color due to anthocyanins, wine color due to copigmentation, lightness (L*), degree of blueness (b*), and chroma (C*).

The feasibility of using FT-MIR spectroscopy for the measurement of grape and wine phenolic composition showed that for commercial wines it was possible to quantify tannins but not the less abundant phenolic classes. In grape extracts and young wines, FT-MIR spectroscopy allowed quantification of total phenols and tannins, and only to some extent anthocyanins. Finally, it was found that the FT-MIR spectra of grapes to some extent also allowed prediction of some wine color attributes.

These results thus demonstrated that it is possible to extract a high proportion of the grape phenols with a short solvent contact time. It was also concluded that color attributes of pH normalized wines could be predicted from analysis of the anthocyanin content of the grapes. Finally, it was concluded that it was only possible to some extent to quantify the levels of anthocyanins in grape extracts by FT-MIR spectroscopy. FT-MIR spectroscopy did however allow quantification of tannins and total phenols in grape extracts and wines. However, the correlation between the tannins levels in grapes and wines were poor, and therefore it is difficult to predict wine tannins from grape measurements

The results found in this thesis thus proved the primary hypotheses that were put up for the work. The results hold promise that it may be possible to introduce rapid, objective measurements of grape phenolics in wineries to allow prediction of wine color. FT-MIR also showed good promise regarding tannin quantification in both grapes and wine, which may have industrial applications in for instance evaluating tannin extraction during fermentation or tannin concentration in blending operations. Unraveling the details about extraction, chemical reactions and transformations of the phenols during red winemaking remains a challenging research field for allowing accurate predictions of the phenolic composition and color attributes of wine.

SAMMENFATNING

Den phenoliske sammensætning i rødvin er vigtig for flere af vinens organoleptiske egenskaber, herunder farve, mundfornemmelse, bitterhed og smag. Rødvins farve er en vigtig kvalitetsparameter, og det er påvist at farveintensiteten af rødvin korrelerer positivt med kvalitetsbedømmelsen af rødvin. Rødvins farve skyldes de phenoliske forbindelser, som i overvejende grad stammer fra druerne. Sammenhængen mellem det phenoliske indhold i druer og rødvinens phenoliske indhold og farve-egenskaber er dog kompleks. Samtidig er de eksisterende metoder til at ekstrahere phenoler fra druer både arbejds- og tidskrævende og derfor ikke anvendelige til rutinemæssige analyser i vinindustrien. Tilvejebringelsen af hurtige metoder til objektivt at analysere det phenoliske indhold i røde druer kan være en værdifuld støtte til at foretage vigtige beslutninger i vinindustrien, f.eks. bestemmelse af høsttidspunkt, prisfastsætning af druer, optimale bearbejdningsbetingelser, og segregering af druer. De følgende hypoteser ligger hermed til grund for dette PhD projekt:

- Det er muligt at definere en robust og generel protokol til hurtigt at ekstrahere de phenoliske forbindelser fra røde druer.
- Det er muligt at prædiktere rødvins farveegenskaber og phenoliske indhold fra det phenoliske indhold af de tilsvarende druer.
- Det er muligt at anvende midt-infrarød spektroskopi til at måle det phenoliske indhold af røde druer.

Formålet med PhD projektet har dermed været at undersøge prædiktionen af rødvins farve-egenskaber fra det phenoliske indhold af druer. Dette indebar udviklingen af en hurtig protokol, til at ekstrahere drue phenolerne og en undersøgelse af muligheden for at anvende Fourier transformeret midt-infrarød (FT-MIR) spektroskopi til at analysere det phenoliske indhold af druer og vin.

Effekten af adskillige faktorer på ekstraktionsgraden af total phenoler og anthocyaniner fra druer blev vurderet for solvent ekstraktion. Både ekstraktionstemperaturen og solvent indholdet af ethanol og saltsyre havde meget signifikante effekter på ekstraktionsgraden af total phenoler og anthocyaniner. Disse resultater skabte grundlaget for en optimeret ekstraktionsmetode med en høj gennemsnitlig ekstraktionsgrad af total phenoler (81.8 %) og anthocyaniner (91.5 %) fra druehomogenat med kun 5 minutters solvent kontakt tid. Den optimerede ekstraktionsprocedure blev testet på otte forskelle druesorter og konkluderet til at give konsistente resultater for forskellige druesorter.

Sammenhængen mellem den phenoliske sammensætning af druer og vin blev undersøgt via den optimerede ekstraktionsprotokol og eksperimentelt fremstillede vine. Den gennemsnitlige andel af phenoler i vin genfundet fra druerne var lav for total phenoler (0.44), tanniner (0.32) og anthocyaniner (0.31), mellem for (+)-catechin (0.75) og polymeriske pigmenter (0.98) og høj for gallussyre (7.9). Den direkte sammenhæng mellem drue og vin phenoler var god for anthocyaniner ($r = 0.93$), total phenoler ($r = 0.88$), (+)-catechin ($r = 0.95$) og (-)-epicatechin ($r = 0.95$) og mindre god for de andre phenoliske klasser. Anvendelsen af en multivariat fremgangsmåde til at prædiktere det phenoliske indhold i vin fra den detaljerede phenoliske sammensætning af druer gav kun mindre forbedringer. En multivariat fremgangsmåde gav dog en forbedret prædiktion af polymeriske pigmenter i vin, grundet en god korrelation mellem anthocyanin i druer og polymeriske pigmenter i vin ($r = 0.87$).

Det var muligt at prædiktere adskillige farve parametre for pH normaliserede eksperimentelt fremstillede rødvine ud fra druernes phenoliske sammensætning med en multivariat fremgangsmåde. Det viste sig imidlertid, at det var tilstrækkeligt at anvende druernes anthocyanin indhold til at prædiktere vin farve parametrene. Med residual prædiktiv afvigelses (RPD) værdier mellem 2.4 og 5.7 var det muligt at prædiktere følgende farve parametre fra måling af anthocyanin indholdet af druer: Farve intensitet, total vinfarve, vinfarve fra anthocyaniner, vinfarve fra copigmentering, lyshed (L*), graden af blå (b*) og chroma (C*).

Undersøgelsen af anvendelsen af FT-MIR spektroskopi til måling af den phenoliske sammensætning af druer og vin viste, at det var muligt at kvantificere tanniner, men ikke de mindre koncentrerede phenoler i kommercielle rødvine. I drue ekstrakter og unge rødvine var det muligt at kvantificere total phenoler og tanniner med FT-MIR spektroskopi, mens anthocyaniner kun i nogen grad kunne kvantificeres. Ydermere var det muligt at prædiktere flere vinfarve parametre fra FT-MIR spektroskopiske målinger af drueekstrakterne.

Resultaterne viste hermed, at det er muligt at ekstrahere en høj andel af drue phenolerne med en kort solvent kontakt tid. Det blev også konkluderet at adskillige farve parametre af eksperimentelt fremstillede rødvine kunne prædikteres fra anthocyanin indholdet i druer. Endeligt blev det konkluderet, at det kun i nogen grad var muligt at kvantificere anthocyanin indholdet i drue ekstrakter med FT-MIR spektroskopi. Det var dog muligt at kvantificere tannin indholdet i drue ekstrakter og rødvin med FT-MIR spektroskopi. En dårlig korrelation mellem tannin indholdet i drue ekstrakter og vin besværliggjorde dog prædiktionen af tannin indholdet af vin fra drue målinger.

Resultaterne i denne afhandling har dermed eftervist de fremsatte hypoteser. Resultaterne ser lovende ud med henblik på at introducere hurtige og objektive målinger af drue phenoler til at forudsige farve parametre i rødvin. Resultaterne var også lovende med henblik på at anvende FT-MIR spektroskopi til at kvantificere tannin i rødvin og druer. Sådanne tannin målinger vil f.eks. kunne finde anvendelse til at vurdere ekstraktionen af tanniner under vinfremstillingen og tannin niveauerne i vine når de skal blandes med andre vine. Tilvejebringelsen af et bedre kendskab til detaljerne omkring ekstraktion, kemiske reaktioner og omdannelser af phenolerne under fremstillingen af rødvin er dog stadig en udfordring.

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Jensen, J.S., Blachez, B., Egebo, M., and Meyer, A.S. 2007. Rapid Extraction of Polyphenols from Red Grapes. Am. J. Enol. Vitic. 58:451-461.

Paper II
Jensen, J.S., Werge, H.H.M., Egebo, M., and Meyer, A.S. 2008. Effect of Wine Dilution on the Reliability of Tannin Analysis by Protein Precipitation. Am. J. Enol. Vitic. 59:103-105.

Paper III
Jensen, J.S., Demiray, S., Egebo, M., and Meyer, A.S. 2008. Prediction of Wine Color Attributes from the Phenolic Profiles of Red Grapes (*Vitis vinifera*). J. Agric. Food Chem. 56:1105-1115.

Paper IV
Jensen, J.S., Egebo, M., and Meyer, A.S. 2008. Identification of Spectral Regions for Quantification of Red Wine Tannins with Fourier Transform Mid-Infrared Spectroscopy. J. Agric. Food Chem. Accepted for publication.

CHAPTER 1 INTRODUCTION AND HYPOTHESES

1.1.1 Background

An important prerequisite for producing high quality wines is that the quality of the grapes is also high. Grape quality and thus the potential of the grapes to give a high quality wine is both evaluated subjectively (e.g. taste, aroma, and visual inspections) and objectively from compositional analyses of the grapes (Krstic et al. 2003). The results provide the base for making important decisions regarding e.g. the optimal harvest time, pricing of the grapes, segregations of grapes, and optimal processing conditions.

The ripening of grapes is associated with accumulation of sugars in the juice and a decreasing acidity. Hence total soluble solids and acidity of the grapes have traditionally been used for evaluating the maturity of the grapes, both with regards to determining the harvest time and with respect to deciding the price of the grapes. However the sugar content of the grapes has only little impact of the final quality of the wine and in warmer regions it is often easy to reach the desirable sugar levels in the grapes (Gishen et al. 2002). Knowledge of other components which are related more directly with wine quality parameters would therefore be useful in the evaluation of grape quality. In addition, if the relationship between the grape and wine composition can be modeled it should be possible to allow prediction of important wine quality parameters from grape measurements. Such prediction could be a valuable support for the decisions concerning harvesting time, payment, processing conditions, and segregation.

It has long been recognized, that phenolic compounds are important for several organoleptic properties of wine, such as wine color, mouthfeel properties, flavor, and bitterness (Gawel 1998, Kennedy et al. 2006b, Preys et al. 2006, Vidal et al. 2004). The color of a red wine is one of the first impressions of the wine and is thus an important quality parameter. It has been reported, that color intensity, at least to some extent, correlates directly with the perceived quality of red wine (Jackson et al. 1978, Somers and Evans 1974). More recently it was also found that wine color intensity correlated with both flavor intensity and wine quality score of wines made from Shiraz grapes (Gishen et al. 2002).

The color of red wines depends largely on its phenolic composition, notably the levels of anthocyanins, polymeric pigments, and anthocyanin derived pigments (Cheynier et al. 2006, Fulcrand et al. 2006, Somers 1971). Thus evaluation of the phenolic composition of the grapes may allow a more direct evaluation of the quality of grapes. Although the phenolic compounds in wine originate from the grapes, the relation between grape and wine phenols is complicated due to several factors. Extraction of the phenols from the grapes into the fermenting must is an incomplete process, which rarely extracts more than 50 % of the phenols from the grapes. In addition the phenols are reactive compounds, and will continuously undergo several chemical changes during the entire winemaking process, including condensation reactions with other phenols. Such reactions impact the wine color. Wine color is also highly affected both by pH and sulfite levels, but also by the presence of non-colored compounds, in particular other phenols, that can enhance the color by molecular associations with the pigments. Since wine color not only relates to the level of anthocyanins in grapes, the

establishment and use of a multivariate relation between the detailed phenolic composition of grapes and wine color could improve the understanding of the relation between the phenolic composition of grapes and the wine color. Knowledge of the phenolic composition of grapes could also allow prediction of the phenolic composition of wines, and thus indicate certain characteristics of the wine.

FOSS manufactures a purpose-built mid infrared instrument (Winescan ^{TM}) for the wine industry. The Winescan allows fast analysis of the chemical composition of grape juice, must under fermentation, and finished wine with only little or no sample preparation. Several important parameters of the grapes (including sugars, organic acids, potassium, and grape soundness index) can be determined simultaneously from the infrared spectra of the juice. However, the instrument does not currently include analysis of the phenolic composition of the grapes.

One specific obstacle for analysis of the phenolic composition of the grapes by mid infrared spectroscopy is that the phenolic compounds are located in the solid parts of the grapes and thus needs to be extracted prior to analysis. The typical protocols for extraction of phenolics from grapes are both time-consuming and labor intensive, and thus not compatible with routine analysis at the winery. The development of a fast protocol for extraction of the phenols from grapes would be an important step towards using mid infrared spectroscopy for the measurement of the phenolic composition of grapes. Another critical step is to find out how well mid infrared spectroscopy can actually measure the phenols, which are present in quite low concentrations in both grapes and wines (typically below 5 g/L).

1.1.2 Research hypotheses and experimental strategy

The objectives of the present study have been to investigate the prediction of red wine color attributes from the levels of polyphenols in the corresponding grapes and the feasibility of mid infrared spectroscopy for the measurement of the phenolic composition in grapes. The project builds on the following hypotheses:

- It is possible to define a robust general protocol for fast extraction of the phenolic compounds from grapes.
- It is possible to predict color attributes and phenolic compositions of red wine from the phenolic composition of grapes.
- It is possible to use mid infrared spectroscopy to measure the phenolic composition of red grapes.

The experimental strategy for this study was to investigate the influence of important factors on the extraction of phenolic compounds from grapes to develop a fast extraction protocol. To allow a proper evaluation of the phenolic composition of the grapes, it was decided to develop a protocol that extracted a consistent, representative fraction of the phenols from different grape cultivars.

The developed extraction protocol was used for investigating the relation between grape and wine phenols and for the prediction of wine color from the phenolic composition of the grapes. For this purpose experimental wines were produced in microscale from grapes covering a wide range of varieties. Since the extraction of phenols occurs in the maceration period, it was decided to focus on relating the grape phenols with the phenolic composition and color attributes of freshly fermented wines. Due to chemical reactions of phenols and color enhancing properties of some

non colored phenols, it was decided to use a multivariate approach for relating the grape phenols and wine color. These results were used to evaluate if different red wine color attributes could be predicted from the phenolic composition of grapes.

Finally, the feasibility of using mid infrared spectroscopy for measurement of phenolic compounds was both evaluated on grape extracts, experimental wines and commercial wines. Due to the low concentration of phenols in grapes and wines, we focused on measuring the major phenolic components, in particular tannins and anthocyanins.

CHAPTER 2 THEORY: POLYPHENOLS AND WINE COLOR

2.1 Phenolic composition of grapes and wines

Levels of total phenols in red wine grapes typically range between ~ 2 and 11 g/kg (Jackson 1994b, Singleton 1966). In red wines levels of total phenols are typically between ~ 0.8 and 4 g/L and the extraction of total phenols from grapes to wine thus rarely exceeds 50 % and thus explain the different levels in grapes and wines (Haslam 2005).

The amount of total phenols from the different parts of the red grape berry have been estimated to be ~33 % in the skins, ~62 % in the seeds, ~1 % in the pulp, and ~4% in the juice (Zoecklein et al. 1995). Thus polyphenols are mainly present in the skins and seeds of the grape berry with only very small amounts of phenols in the pulp and juice of the grapes.

2.1.1 Classes of phenols in grapes and wines

Due to the large diversity of the phenolic compounds found in grapes and wines, the phenols are commonly classified in more general phenolic classes. A large proportion of the phenols in red wines contain a flavonoid ring structure (Figure 1), and phenols are thus commonly classified as either flavonoids or nonflavonoids.

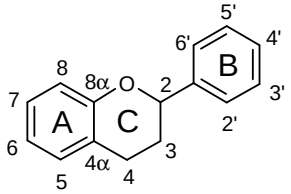


Figure 1. Flavonoid ring structure including numbering of the carbon atoms.

The flavonoids are substituted with e.g. hydroxyl, methoxyl, acyl, or glycosyl groups at different positions and may have double bonds or a carbonyl group in the C ring. The most abundant flavonoid classes in grapes and wines are anthocyanins, flavanols, tannins, and flavonols – these compounds all have OH-substitutions in position 5 and 7 of the A-ring in the flavonoid ring structure, and, as will be discussed below, mainly vary in their hydroxylation pattern and other substitution pattern in the B and C rings.

Anthocyanins

Anthocyanins are responsible for the red color of grapes and young wines and are with only few exceptions exclusively located in the skins of the grapes. In *Vitis vinfera* grapes anthocyanins exist as 3-monoglucosides of the five anthocyanidins: cyanidin, delphinidin, peonidin, petunidin, and malvidin (Figure 2). The glucose part of the anthocyanins can both be unsubstituted or acylated as esters of acetic acid, *p*-coumaric acid, or caffeic acid. Although the anthocyanin profiles can vary highly between grape varieties, malvidin-3-glucoside is typically the most predominant anthocyanin. Reported levels of anthocyanins in red grapes range from 300 to 7500 mg/kg, but the levels vary highly according to the cultivar, maturity, production year and environmental conditions (Mazza 1995). The concentration of anthocyanins in wines varies highly according to the age of the wine and the variety. In young wines anthocyanins range from about 100 mg/L to 1500 mg/L (Ribéreau-Gayon et al. 2006). Anthocyanins are highly reactive compounds and the concentration decreases rapidly

as wine ages and reach levels of as little as 0-50 mg/L in aged wines (Ribéreau-Gayon et al. 2006).

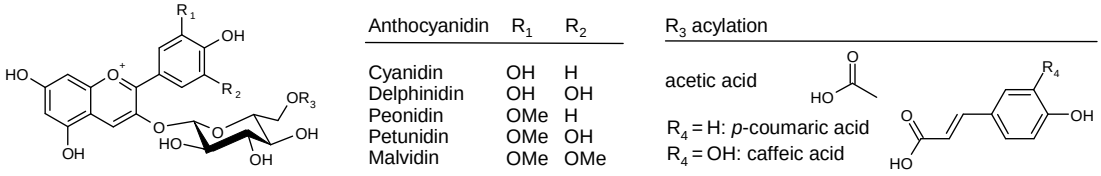


Figure 2. Chemical structures of flavylium form of anthocyanins found in *Vitis vinifera* grapes.

The differences in the substitutions on the B ring of the anthocyanins result in differences in their color properties. The di-substituted anthocyanins (Cy3G and Pn3G) have absorbance maxima at slightly lower wavelengths than the tri substituted anthocyanins (Dl3G, Pt3G, and Mv3G), and thus have a slightly more orange tone in the color (Cabrita et al. 2000). Some authors have reported that the molar absorptivities of the different anthocyanins vary by sometimes a factor of two between the different anthocyanins (Cabrita et al. 2000, Giusti et al. 1999). However, it was recently found that only minor differences (~ 10%) existed between the molar absorptivities of the different anthocyanins and that the previously reported variations may be due to impurities in the anthocyanin samples (Jordheim et al. 2007). Average molar absorptivities were reported to be 22000 L/(cm·mol) for anthocyanidin-3-monoglycosides in aqueous solution at pH 1.

Flavonols

In grapes, flavonols (Figure 3) are present as the glycosylated forms of the parent flavonols (mainly quercetin, myricetin, and kaempferol) and are located primarily in the skins (Castillo-Munoz et al. 2007, Ribéreau-Gayon and Glories 1987). Due to hydrolysis of the glycoside bond during wine production, also unglycosylated flavonols are found in wines. The flavonols are yellow pigments, but may like many other phenolic classes also enhance wine color by copigmentation, which will be discussed in more detail in section 2.2.1 (Schwarz et al. 2005). The total levels of flavonols was recently reported to be between 129 and 346 µmole/kg grapes (corresponding to between 80 and 210 mg rutin equivalents/kg) covering 7 different red grape cultivars and between 28 and 377 µmole/L (corresponding to 17-230 mg rutin equivalents/L) in red wine from 10 different grape cultivars (Castillo-Munoz et al. 2007).

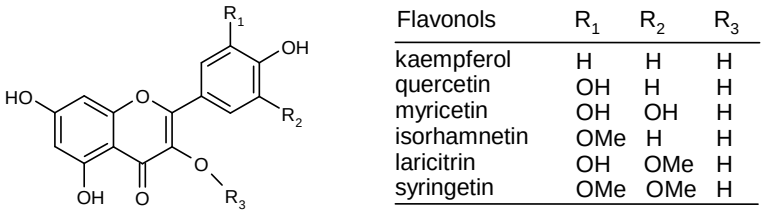


Figure 3. Chemical structures of flavonols found in wine. In grapes the flavonols primarily exist as glycosides (R_3 = sugar residue), e.g. for rutin R_3 = rhamnosylglucoside.

Monomeric and oligomeric flavanols

The flavanols (+)-catechin and (-)-epicatechin are the most common monomeric flavanols and are mainly found in the seeds and skins of the grapes (Figure 4). The less abundant flavanols (+)-gallocatechin and (-)-epigallocatechin are located in the skins of the grapes (Gonzalez-Manzano et al. 2004). Typical levels of monomeric

flavanols in commercial red wines between 30 and 100 mg/L have been reported (Arts et al. 2000).

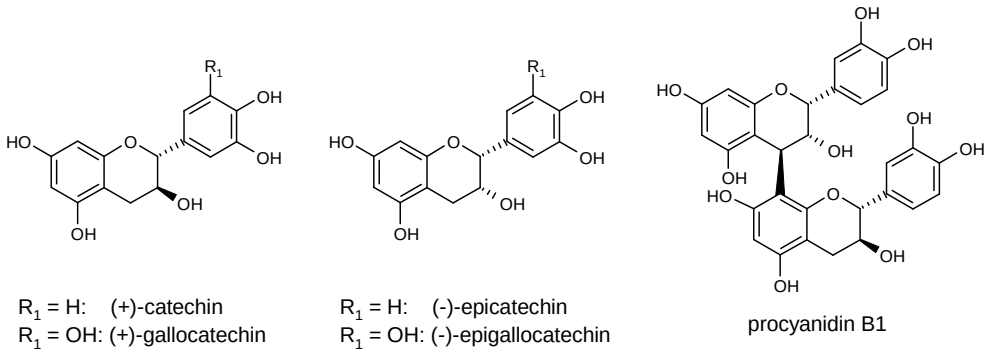


Figure 4. Chemical structures of monomeric flavanols and a dimeric flavanol (procyanidin B1).

Oligomeric flavanols are made up of the building blocks of the monomeric flavanols as illustrated for procyanidin B1 (Figure 4). Numerous oligomeric flavanols have been identified in grapes and wines and the term oligomeric is typically used for flavonols consisting of two to five monomeric units (Monagas et al. 2005). Flavanols contribute both to bitterness and astringency in wines (as well as in grapes) (Peleg et al. 1999).

Tannins

Tannins are the most abundant class of phenols in grapes and red wines (Kennedy et al. 2006b), and play important roles in the color stability and mouthfeel properties of wines (Gawel 1998, Kennedy et al. 2006a, Singleton and Trousdale 1992). The ability of tannins to bind with proteins present in saliva is highly associated with the astringent sensation of red wine (Gawel 1998). Tannins are typically classified as either condensed tannins or hydrolyzable tannins (Figure 5). Condensed tannins are oligomeric and polymeric compounds composed of flavanols units and originate primarily from the seeds and the skins of grapes, while hydrolyzable tannins mainly originate from oak and are gallic acid or ellagic acid esters of glucose (Edelmann and Lendl 2002, Herderich and Smith 2005).

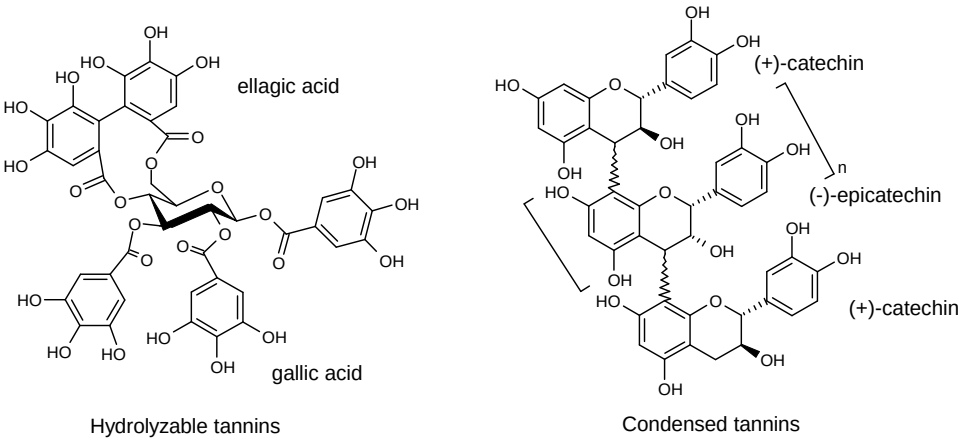


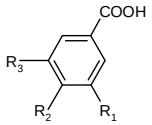
Figure 5. Examples of chemical structures of condensed and hydrolyzable tannins.

Reactions between tannins and anthocyanins during aging of wines leads to more stable pigments, and these conjugates have been suggested to be at least partially responsible for the stability of red wine color (Cheynier et al. 2006). The levels of tannins in red wines measured by protein precipitation (Harbertson et al. 2003) have

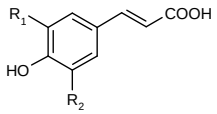
been reported to vary highly from as low as 30 to more than 1500 mg catechin equivalents /L (Fernandez and Agosin 2007, Harbertson 2003, Skogerson et al. 2007, Versari et al. 2006). Tannin levels measured by other methods may give quite different results, according to the specificity of the method towards tannins and the units used to report the results. Overviews of different methods for tannin quantification is found elsewhere (Herderich and Smith 2005, Makkar 1989, Schofield et al. 2001).

Nonflavonoids phenols

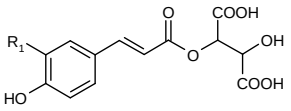
The most abundant group of nonflavonoid phenols found in grapes and wines is the phenolic acids and their derivates (Figure 6). The predominant phenol acids in grapes are the tartrate esters of the hydroxycinnamates, which are mainly found in the pulp and skin of the grape (Adams 2006, Monagas et al. 2005). Considerable amounts of gallic acid and the free hydroxycinnamates will also be present in wines, due to hydrolysis of esterified phenolic acids (Ribéreau-Gayon et al. 2006). Typical levels of phenolic acids are between 100 and 200 mg/L in red wines (Ribéreau-Gayon et al. 2006).



Benzoic acid	R_1	R_2	R_3
p-hydroxybenzoic acid	H	OH	H
protocatechuic acid	OH	OH	H
vanillic acid	OMe	OH	H
gallic acid	OH	OH	OH



Hydroxycinnamates	R_1	R_2
p-coumaric acid	H	H
caffeic acid	OH	H
ferulic acid	OMe	H
Sinapic acid	OMe	OMe



Hydroxycinnamic tartaric esters	R_1
Cutaric acid	H
Caftaric acid	OH
Fertaric acid	OMe

Figure 6. Chemical structures of phenolic acids found in grapes and wines.

A considerable proportion of the nonflavonoid part of the wine phenols may originate from the use of oak during wine aging (Zoecklein et al. 1995). Other classes of nonflavonoid phenols include stilbenes and volatile phenols, which are important for the aroma and antioxidant properties of wines, but are present in very low amounts (Monagas et al. 2005).

2.2 Red Wine Color

2.2.1 Chemistry of red wine color

Anthocyanins

Anthocyanins are mainly responsible for the red color of grapes and very young red wines, due to the highly colored flavylium cation (Figure 7). The flavylium cation exists in equilibrium with both the colorless hemiketal and the blue quinonoidal base, via hydration and proton transfer respectively. Furthermore the red flavylium cation will readily react with bisulfite, resulting in a colorless bisulfite adduct (Berke et al.

1998).

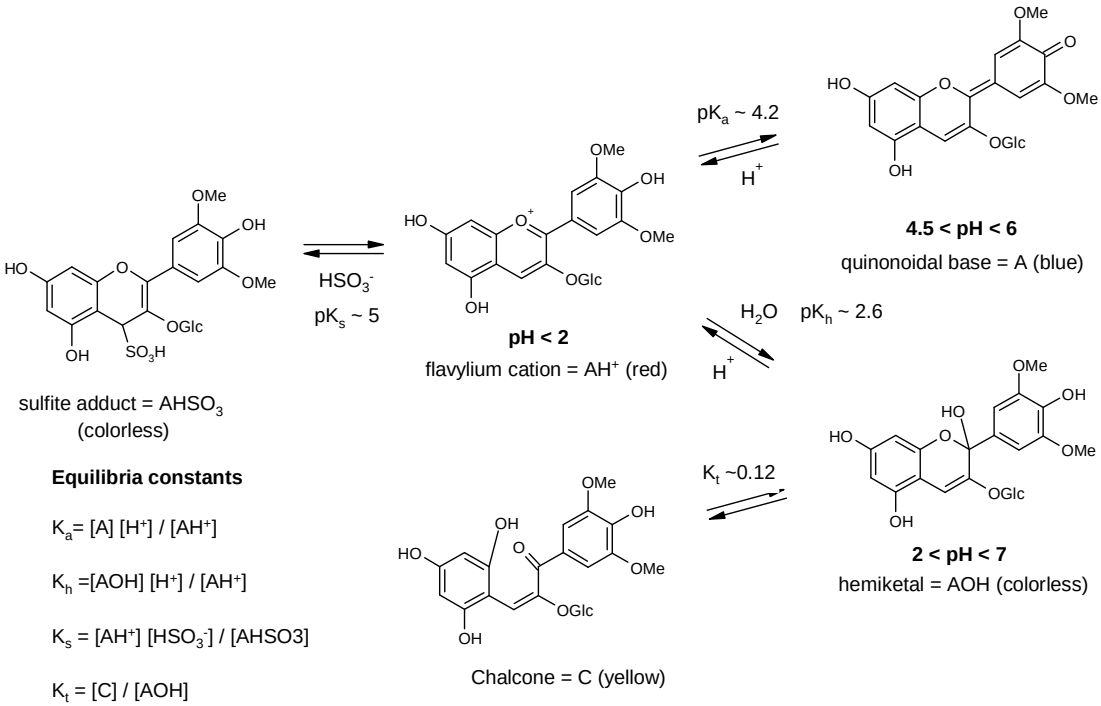


Figure 7. Equilibria between the different forms of malvidin-3-glucoside. Adapted from (Cheynier et al. 2006, Fulcrand et al. 2006).

The equilibria constants for hydration (pK_h ~2.6) and proton transfer (pK_a ~4.2) for malvidin-3-glucoside have been previously estimated (Brouillard et al. 1978, Brouillard and Delaporte 1977, Brouillard and Dubois 1977). From these equilibrium constants it is estimated, that at normal wine pH (pH ~3.6) only 9 % are in the red flavylium form (assuming no bleaching by bisulfite), while the major part are in the hemiketal form (Fulcrand et al. 2006). When bisulfite is present it will readily bind with the flavylium cations and form an anthocyanin sulfite adduct. The formation of the anthocyanin sulfite adduct is very favorable at wine pH, with a dissociation constant pK_s ~ 5 (Fulcrand et al. 2006). Thus anthocyanins are easily bleached with bisulfite.

Copigmentation

Absorbance readings of diluted red wines have been reported to deviate from Lambert-Beers law (Somers 1987), in particular for young wines (Figure 8). The absorbance readings of the concentrated wines are higher than expected from the color of the diluted wines and this observation has been ascribed to the so called copigmentation phenomena (Boulton 2001).

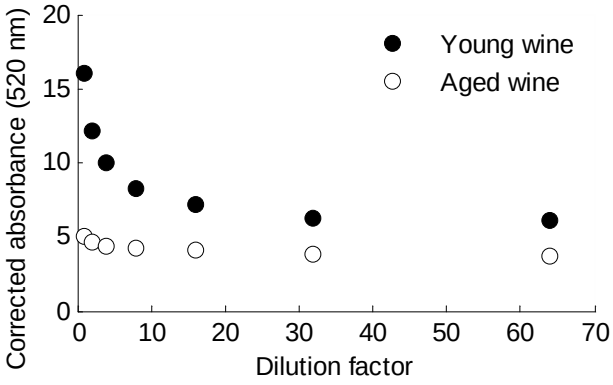


Figure 8. Effect of dilution on the corrected absorbance values of wines (expressed as absorbance values for undiluted wines) at pH 3.7. The young wine contained 430 mg/L anthocyanin and the aged wine 30 mg/L. Graph is adapted from (Somers 1987).

Copigmentation can be defined as the enhancement of color due to molecular associations between pigments and other organic molecules (copigments). The equilibria between the colored and non colored forms of anthocyanins are affected by copigmentation, which stabilize the red flavylium form by complexation and thus allows a higher percentage of the anthocyanins to be in the colored flavylium form. Although the mechanisms behind copigmentation are not fully understood, the current evidence suggest that the complexes between pigments and copigments are organized in planar stacks due to a combination of hydrophobic and π-π interactions between the compounds (Boulton 2001). Besides increasing the absorbance values, copigmentation may also lead to an increase in the wavelength of maximum absorption and thus shift the hue towards the blue and purple tones.

The color enhancement depends on several factors, including concentrations and the chemical nature of the pigment and copigment, the pH of the solution, and the ethanol concentration (Boulton 2001, Gutierrez 2003). A wide range of organic compounds, including phenols typically found in wine (e.g. phenolic acids, flavanols, flavonols) have been found to have copigmentation effects on anthocyanins (Asen et al. 1972). It was recently shown that catechin, epicatechin, and procyanidin were much less effective as copigments to malvidin-3-glucoside than the flavonols quercitrin and myricitrin (which are quercetin and myricetin glycosylated with rhamnose), all in 1:1 molar ratios (Gomez-Miguez et al. 2006). The most abundant class of phenols in red wine, the oligomeric and polymeric flavanols, have also been concluded to be poor copigments(Boulton 2001).

Anthocyanins are themselves good copigments and a considerable proportion of the copigmentation effect can be ascribed to self association of anthocyanins. For instance, it was shown that the color of cyanidin 3,5-diglucoside at pH 3.16 increased twenty fold with only a 10 fold concentration increase from 0.5 mM to 5 mM (Asen et al. 1972). Self association have been reported to be important for anthocyanin concentrations above 1 mM (Boulton 2001).

Copigmentation is very important for the color of young wines and has been reported to account for between 30 and 50 % of the color in young red wines (Boulton 2001, Levengood and Boulton 2004, Mazza et al. 1999). As wine ages the copigmentation effect decreases and is thus not as important for aged wines. It was recently shown

that the color due to copigmentation decreased from between 32 and 44 % to between 0 and 5 % during 9 months of aging (Gutierrez et al. 2005).

Anthocyanin derived pigments

Anthocyanins are highly reactive compounds and will react with other compounds, present in the wines such as acetaldehyde, tannins, keto acids, and cinnamates to produce pigments, which in many cases have different color properties with regards to changes in pH and sulfite additions (Harbertson and Spayd 2006). Pigments that resist bleaching with bisulfite have traditionally been classified as polymeric pigments, and it has been reported that polymeric pigments can account for up to 50 % of the color in one year old wines (Somers 1971). However, it has been demonstrated that the term polymeric pigment is somewhat misleading, since both some pigments of polymeric nature are bleached and some monomeric pigments (e.g. pyranoanthocyanins) are not bleached by bisulfite.

The conversion of anthocyanins into other pigments and non colored compounds can take place via different routes. Examples of anthocyanin derived pigments found in wines are illustrated in Figure 9.

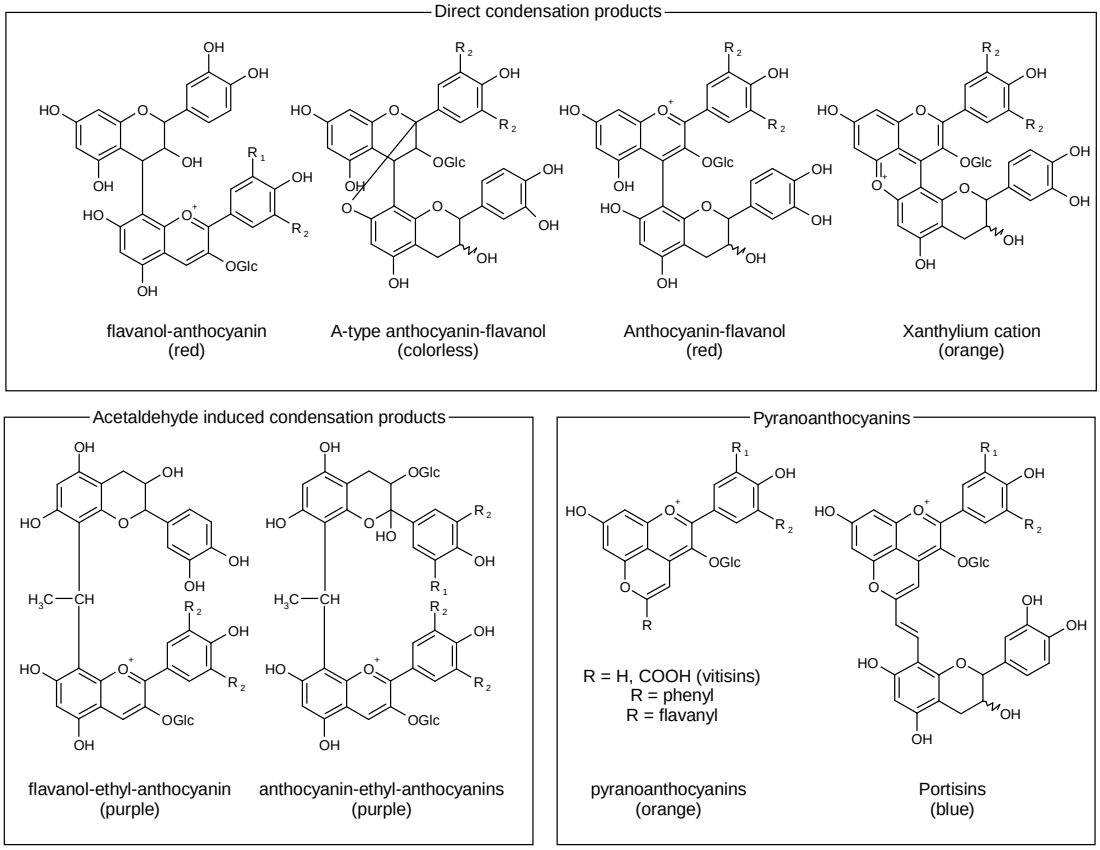


Figure 9. Examples of chemical structures of anthocyanin derived pigments. Glc = glucose and R_1, R_2 = H, OH, or OMe. Figure is modified from (Cheynier et al. 2006, Fulcrand et al. 2006).

Both direct condensations and acetaldehyde mediated condensations between anthocyanins and flavanols lead to new anthocyanin flavanol conjugates (Remy et al. 2000, Salas et al. 2005). The flavanol can be both monomeric and polymeric and thus produce small pigments and polymeric pigments from the condensation.

Condensation products between two anthocyanin compounds have also been detected in wines (Salas et al. 2005). Anthocyanins will also react with yeast metabolites and vinyl phenols to produce pyranoanthocyanins (Fulcrand et al. 2006). The color properties of the anthocyanin derived pigments at wine pH range from the colorless A-type adducts, over the orange pyranoanthocyanins, to the purple acetaldehyde condensation products and finally the blue portisins (Cheynier et al. 2006). In addition to color shifts, the pigments also react differently towards pH changes and bisulfite additions. Pyranoanthocyanins remain colored under a wide range of pH values and bisulfite levels, since the substitution of the middle ring prevents nucleophilic attack of water or sulfites (Cheynier et al. 2006). The flavanol-anthocyanin pigment catechin-malvidin-3-glucoside exhibit similar reactivity towards pH changes and sulfite additions as monomeric anthocyanins (Salas et al. 2004).

The changes in the different pigment concentrations with the age of red wine was recently reported (Boido et al. 2006). The total concentration of wine pigments rapidly decreased as the wine aged and after 64 months of aging pyranoanthocyanins were present in higher amounts than anthocyanins in the wines. The contribution of various pigments to the overall color of wines were evaluated for polymeric pigments, malvidin-3-glucoside, and vitisin A in aged red wines (Schwarz et al. 2003). The color contribution from polymeric pigments amounted to between 70 and 90 % of the overall wine color, while less than 5 % of the color was ascribed to vitisin A. This indicated the importance of polymeric pigments for the color of aged red wine.

Oxidation of phenols

Both enzymatic and non-enzymatic oxidation of phenols will readily occur when must or wine is exposed to oxygen. Oxidation will have an impact on wine color and typically leads to browning of the wine (Zoecklein et al. 1995).

Enzymatic oxidation occurs primarily when the grapes are crushed, with polyphenol oxidases (from the grapes) and laccase (from fungal growth) being the responsible enzymes. Oxidation of phenols with polyphenol oxidases (EC 1.10.3.1 and EC 1.18.18.1) occurs only for phenols with two adjacent phenol groups, while laccase (EC 1.10.3.2) may also oxidize other phenols (Jackson 1994b). The formed quinones will react with themselves or other phenols leading to brown polymeric products. The quinones can also react with other non phenolic compounds, for instance glutathione is known react with the oxidation product of caftaric acid (Singleton et al. 1985). Furthermore enzymatic oxidation of anthocyanins have been demonstrated to occur as a coupled oxidation process of *ortho*-diphenols via such quinones (Yokotsuka and Singleton 1997).

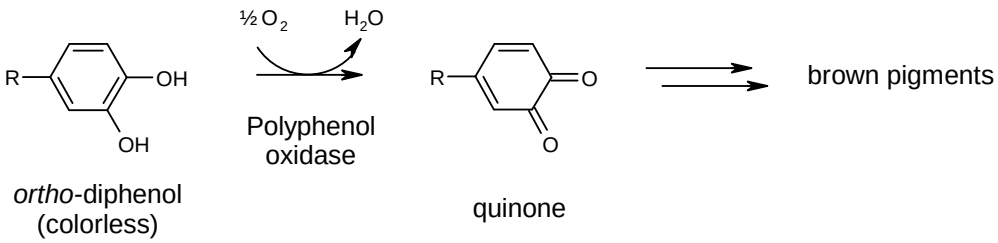


Figure 10. Enzymatic oxidation of *ortho*-diphenols into quinones, which will react with other phenols to produce brown pigments. Adapted from (Zoecklein et al. 1995).

Direct reactions between oxygen and phenols are unfavorable at wine pH. Instead it has been proposed that chemical oxidation occurs via reactive oxygen species such as the hydroperoxyl radical (HOO·), which are formed catalytically in the presence of e.g. iron or copper (Waterhouse and Laurie 2006). Chemical oxidation is favored for phenols having adjacent phenolic groups leading to the formation of quinones. Chemical oxidation of other compounds than phenols is also important for wine color, since e.g. oxidation of ethanol to acetaldehyde is important for the formation of ethyl linked anthocyanin derivates and pyranoanthocyanins (Fulcrand et al. 2006).

2.2.2 Measurement of red wine color

The color of a red wine gives the first impression of the wine and is an important quality parameter of red wine. Therefore there has long been a desire to measure and control the color of wines. A typical spectrum of a red wine in the visible region has a characteristic peak around 520 nm from the anthocyanins and anthocyanin derived pigments (Figure 11). Several approaches have been taken to describe wine color in a simple and meaningful way from the wine spectra (Harbertson and Spayd 2006).

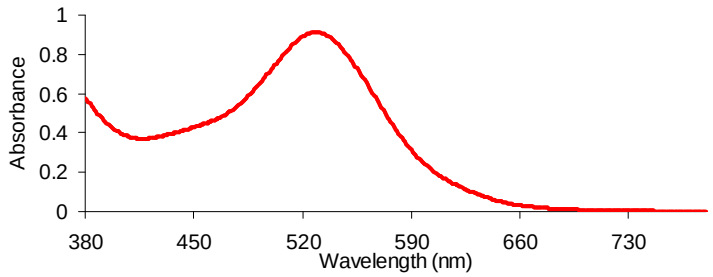


Figure 11. Typical spectrum (1 mm path length) of an undiluted young red wine from 380 to 780 nm (Jensen, unpublished data).

Since the levels of bisulfite and pH in the wines will have a big impact on the color impressions of the wines, it may be difficult to compare the color of wines with different bisulfite and pH levels. To allow a proper comparison of the color of wines, the pH values of the wines are often normalized (Iland et al. 2004). The bleaching effect of bisulfite can be removed by the addition of acetaldehyde, which readily binds with bisulfite and thus frees sulfite bleached anthocyanin adducts.

Color intensity and tonality

Traditionally, the absorbances at 420 and 520 nm have been used to describe the wine color as a combination of the color intensity (A420 + A520) and the tonality or hue (A420/A520) (Sudraud 1958). The color intensity was later extended to also include the absorbance at 620 nm (Glories 1984). The color tonality describes the change of red color towards orange tones occurring during wine aging, while the color intensity describes how much color the wine has (Ribéreau-Gayon et al. 2006). These color measurements are being widely used in the industry due to their simple measurements and ease of interpretation.

Boulton's color assay

Alternatively, wine color can be described according to the contribution of different factors. A recently developed assay allows determination of the total wine color, and the contribution from anthocyanins, polymeric pigments, and copigmentation to the overall wine color (Levengood and Boulton 2004). In this method, wine samples are

adjusted to a fixed pH of 3.6, to eliminate color differences due to pH effects and three separate absorbance readings are performed (Figure 12). The total wine color is measured as the absorbance at 520 nm after removing any bleaching effects from SO_2 with acetaldehyde (A^{acet}). The color due to polymeric pigments is determined from the absorbance after bisulfite bleaching (A^{SO2}). A twenty fold dilution or more of the wine in a model wine solution disrupts copigmentation complexes and this absorbance measurement is used to determine the wine color without interference from copigmentation effects (A^{20}). Color due to copigmentation is calculated as the difference between the undiluted and the diluted absorbance values. The color due to anthocyanins is determined as the difference between the absorbance of the diluted and the bleached wine.

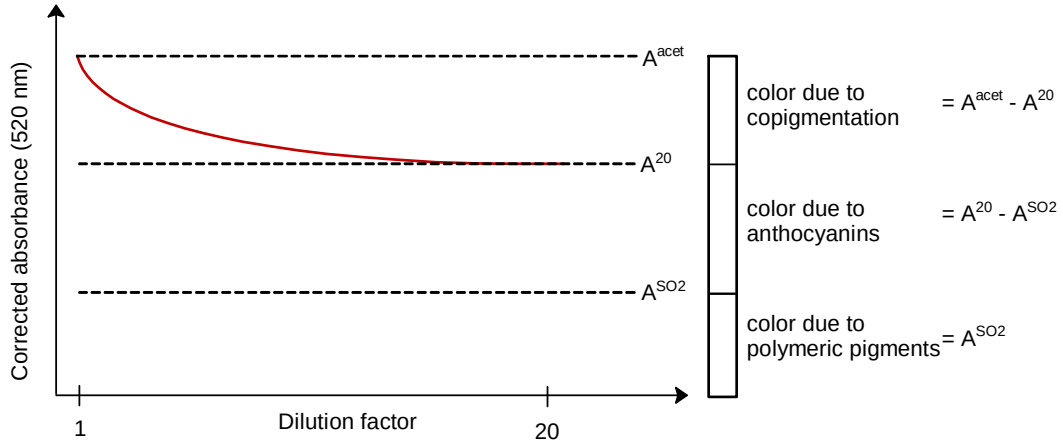


Figure 12. Illustration of Boulton's assay for determination of color due to anthocyanins, copigmentation, and polymeric pigments.

CIE color system

A standard color system for describing color quantitatively has been defined by 'Commission Internationale L'Eclairage' (abbreviated CIE). The color system is widely used in many industries and has also been proposed for applications within the wine industry (Ayala et al. 1997, Perez-Caballero et al. 2003).

The principle of the CIE color system is that any given color can be described as a combination of the tristimulus values X, Y, and Z representing red, green, and blue colors respectively (Ohta and Robertson 2005a). The tristimulus values can be calculated from the transmittance values of the samples across the entire visible spectrum using the following formulas (Ohta and Robertson 2005c):

$$X = k\sum_{380}^{780} T(\lambda)P(\lambda)\bar{x}_{10}(\lambda)$$

$$Y = k\sum_{380}^{780} T(\lambda)P(\lambda)\bar{y}_{10}(\lambda)$$

$$Z = k\sum_{380}^{780} T(\lambda)P(\lambda)\bar{z}_{10}(\lambda)$$

$$k = 100/\sum_{380}^{780} P(\lambda)\bar{y}_{10}(\lambda)$$

Where:
$T(\lambda)$ is the transmittance values of the wine at λ.
$P(\lambda)$ is the spectral distribution of the D65 standard illuminant, representing average daylight.
$\bar{x}_{10}, \bar{y}_{10}, \bar{z}_{10}$ are the color matching functions for the CIE 1964 standard colorimetric observer

Commonly the color is not reported using the tristimulus values, but are instead converted to CIELab color values, which are regarded easier to correlate with the visual appearance of samples (Ohta and Robertson 2005b). Very few studies have

however investigated the relation between CIELab color values and sensory determined color attributes (Martinez et al. 2001, Ortiz et al. 1995) and it still remains to be proven that CIELab color values measured using a spectrophotometer are the best way to describe wine color. The different CIELab parameters with a short description of their meanings are shown in Table 1.

Table 1. Explanation of the CIELab color parameters.

Parameter	Description of parameter
L^*	Lightness from 0 (black) to 100 (white)
a^*	Degree of red/green, where $a^*<0$ is green and $a^*>0$ red
b^*	Degree of blue/yellow, where $b^*<0$ is yellow and $b^*>$ is blue
C^*	Chroma (colorfulness), where grey have chroma = 0
H^*	Hue angle (tone), e.g. red ($H^*=0$), yellow ($H^*=90$), green ($H^* = 180$), and blue ($H^*=270$)

The tristimulus values can then be transformed into the CIELab color values from the following formulas (Ohta and Robertson 2005b):

$$L^* = 116\, f(Y/Y_n) - 16$$

$$a^* = 500\{ f(X/X_n) - f(Y/Y_n)\}$$

$$b^* = 200\{ f(Y/Y_n) - f(Z/Z_n)\}$$

$$C^* = \sqrt{a^{*2} + b^{*2}}$$

$$h^* = \arctan(b^*/a^*)$$

where

$X_n = 94.825$, $Y_n = 100$, and $Z_n = 107.381$ for the D65 illuminant

$$f(X/X_n) = (X/X_n)^{1/3} \quad \text{if} \quad X/X_n > (24/116)^3 \text{ (applies also for Y and Z)}$$

$$f(X/X_n) = (841/108)(X/X_n) + 16/116 \quad \text{if} \quad X/X_n \le (24/116)^3 \text{ (applies also for Y and Z)}$$

Several authors have applied the CIELab color space to characterize the color of red wine, but also with some slightly different methods. The 'Office Internationale de la Vigne et du Vin' (O.I.V.) currently recommends that CIELab color values are calculated from the full transmittance spectra of wines, referred to a 1 cm cuvette path length (O.I.V. 2008).

Several authors have used simplified absorbance measurements to estimate the CIELab parameters, which allowed the use of simpler and less expensive spectrophotometers (Ayala et al. 1999, Perez-Caballero et al. 2003, Pérez-Magarino and Gonzalez-Sanjose 2003). The most accurate model used the transmittance readings at 4 different wavelengths: 450, 520, 570, and 630 nm to estimate the tristimulus values (Perez-Caballero et al. 2003):

$$X = 19.717\tau_{450} + 1.884\tau_{520} + 42.539\tau_{570} + 32.474\tau_{630} - 1.841$$

$$Y = 7.950\tau_{450} + 34.764\tau_{520} + 42.736\tau_{570} + 15.759\tau_{630} - 1.180$$

$$Z = 103.518\tau_{450} + 4.190\tau_{520} + 0.251\tau_{570} - 1.831\tau_{630} + 0.818$$

The tristimulus values were then converted to CIELab color values from the formulas showed above (Ohta and Robertson 2005b).

These authors referred the absorbance readings to 2 mm path lengths for red and rosé wines and 1 cm for white wines. CIELab color values, determined using this method has recently been used to follow the color changes during aging (Gutierrez et al. 2005, Monagas et al. 2006b). Both these studies found, that as the wines aged the chroma decreased, the hue angles increased towards more yellow tones and lightness both decreased and increased.

2.3 Extraction of polyphenols from grapes

2.3.1 Phenolic extraction during winemaking

The basic operation steps in the production of red wines are illustrated in Figure 13. Although all operation steps will have an impact on the final wine, also with regards to the phenolic composition, the focus of the present study is on the extraction of phenols from the grapes during maceration. A general review of the different operations process is found elsewhere (Jackson 1994a).

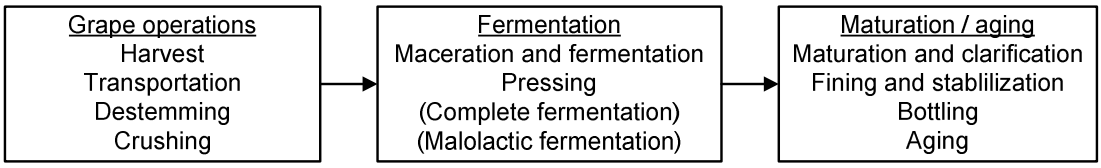


Figure 13. Typical operations in the production of red wine. Adapted from (Jackson 1994a).

Production of red wine requires a maceration step, where the phenolic compounds are extracted from the solid parts into the fermenting must. The concentration of anthocyanins increases rapidly during the few days of fermentation, but after about six days time the anthocyanin concentration reaches a maximum and then starts to decrease (Table 2) (Ribéreau-Gayon and Glories 1987). This decrease has been ascribed to an exceeding rate of conversion of the reactive anthocyanins to other compounds compared with the extraction of the anthocyanins from the grape material in the later stages of maceration (Cheynier et al. 2006). This trend has been corroborated in other studies (Sacchi et al. 2005). In a similar manner color intensity of the wines also reaches a maximum after about six days and then starts to decrease (Ribéreau-Gayon and Glories 1987).

Table 2. Impact of maceration on color intensity, anthocyanins, tannins, and total phenols during vinification, adapted from (Ribéreau-Gayon and Glories 1987).

maceration time (days)	color intensity (abs)	anthocyanins (mg/L)	tannins (mg/L)	total phenols (index)
2	0.89	0.46	1.77	30
3	1.24	0.5	1.96	37
6	1.43	0.67	2.63	48
10	1.41	0.61	3.39	60
20	1.21	0.48	3.65	62
40	1.22	0.38	4.26	70

On the contrary, the levels of total phenols and tannins increases more slowly in the beginning of the maceration, but continues to increase even with very long extraction times of up to 40 days (Ribéreau-Gayon and Glories 1987). This continued increase can be ascribed to a much slower extraction of monomeric flavanols and tannins from the seeds than the skins of grapes (Des Gachons and Kennedy 2003, Gonzalez-

Manzano et al. 2004). Ethanol positively increases the extraction of tannins from grape seeds, and the increasing levels of ethanol in the fermenting must will facilitate tannin extraction from the seeds. The majority of the monomeric and polymeric flavanols are located in the outer seed coat (Thorngate and Singleton 1994) and it has been reported, that under simulated winemaking conditions it is possible to extract the majority of the seed polyphenols (Singleton and Draper 1964).

2.3.2 Grape extraction for phenolic analysis

Phenolics are primarily located in vacuolar structures in the solid parts of the grape and thus needs to be extracted in a suitable solvent to allow analysis of the phenolic composition. Typically, extraction is carried out by a solvent extraction, where the sample is first grinded to reduce particle size and then steeped with a suitable solvent (Escribano-Bailon and Santos-Buelga 2003). Several factors influence the efficiency of the solvent extraction and may be critical for the analytical results.

Sample preparation

Sample preparation for phenolic extraction may both involve reduction of the particles size and drying of the sample. For grapes a large proportion of the phenols are found in the seed, and it has been found that extraction of seed phenols are greatly facilitated by crushing of the seeds (Meyer et al. 1997). The freezing of grapes have on the other hand been reported to ease the phenolic extraction in winemaking, likely due to disruption of the cells from the freezing (Jackson 1994b, Sacchi et al. 2005). In laboratory settings it is common to freeze dry samples to remove water and grind the dry material to obtain a powder of small particle sizes. Such an approach has been reported for phenolic analysis of separated skins and seeds grapes (Montealegre et al. 2006). A simpler approach involves a thorough crushing of fresh or frozen grapes using a high speed blender homogenizer prior to extraction with aqueous ethanol (Iland et al. 2004). Consistent analytical results were found for both fresh and frozen grape material and three different homogenizers for anthocyanins and total phenols (Cynkar et al. 2004).

Extraction solvent

Extraction of phenols from plant material is typically carried out using an organic solvent of which acetone, methanol and ethanol are the most used (Cheynier 2006). Extraction of flavanols, which constitute a major part of the seed phenolics, are in general more difficult to extract than anthocyanins (Escribano-Bailon and Santos-Buelga 2003). A survey of different solvents for the extraction of flavanols from seeds showed that 70 % aqueous acetone was very good for the extraction of oligomeric flavanols, while methanol extracted the monomeric flavanols very well and 70 % aqueous ethanol extracted gallic acid very well (Kallithraka et al. 1995). For acetone, methanol, and ethanol higher extraction yields are obtained when aqueous mixtures between 50 and 70 % are used (Yilmaz and Toledo 2006). The highest extraction yields of total phenols from grape seeds were found to be ~28 mg gallic acid equivalents/g seed for 60 % ethanol, ~32 g gallic acid equivalents/g seed for 70 % methanol, and ~42 mg gallic acid equivalents/g seed for 50 % acetone (Yilmaz and Toledo 2006).

Acidified solvents are commonly used for anthocyanin extractions, but high levels of acid in the solvents may lead to degradation of acylated anthocyanins (Revilla et al. 1998). The presence of acid has been reported to facilitate the extraction of

anthocyanins due to a disruption of the plant cell membranes and thereby releasing the phenols (Ribéreau-Gayon et al. 2006). This principle has been used to evaluate the extractability of anthocyanins in red grapes as an indicator of the phenolic maturity of the grapes (Saint-Cricq De Gaulejac et al. 1998). The method consist of comparing the extraction of anthocyanins under simulated wine making condition (pH 3.2 or pH 3.6), but require a four hour long extraction.

Extraction temperature, time and ratio of solvent to solid

A recent study on the extraction of phenols from grape byproducts have shown, that increased extraction temperatures (50 ºC), long extraction times (90 minutes) and high ratios of extraction solvent to solid (5:1) increases the phenolic yields for both methanol and ethanol extractions (Pinelo et al. 2005). Heat impacts the extraction efficiency of phenols by increasing the permeability of the cell membrane and the solubility and diffusion of the phenols (Escribano-Bailon and Santos-Buelga 2003). A long extraction time is important for the diffusion of phenols from the grape material to complete. A high solvent to solid ratio facilitates extraction due to an increased concentration gradient from the solids to the solvent, which is the driving force for extraction kinetics (Pinelo et al. 2005).

Reported methods for extraction of phenols from grapes

Table 3 gives an overview of the most relevant methods for the extraction of phenols from grape material. The almost complete extraction of anthocyanins reported by Iland et al. is a widely used reference method for the analysis of grape color in the Australian wine industry (Iland et al. 2004).

Table 3. Overview of selected protocols for the extraction of phenols from grapes

Reference	Extracted material	Solvent	Extraction time	Extraction temperature	Ratio of solvent to solid
(Iland et al. 2004)	Grape homogenate	50 % ethanol (pH 2)	1 hour	R.T.	10:1
(Saint-Cricq De Gaulejac et al. 1998)	Grape homogenate	12 % ethanol (parallel extractions at pH 1 and 3.2)	4 hours	R.T.	1:1
(Mane et al. 2007)	Separated skin, pulp, and seeds	51:34:15:0.05 % acetone:H_2O: methanol:TFA	67-90 minutes	R.T.	at least 50:1
(Kennedy et al. 2000)	Grape seeds	66 % acetone	24 hours	R.T.	1:1 [b]
(Revilla et al. 1998)	Whole grapes grinded in contact with solvent	100% methanol (three step extraction)	4 hours, 12 hours, 4 hours	R.T.	0.60 ml solvent per grape

[a] TFA = trifluoroacetic acid. [b] Ratio of solvent to original grape mass

Glories' extraction method has been used to evaluate the extractability of anthocyanins, which provides a good control of the optimal harvest time (Saint-Cricq De Gaulejac et al. 1998). Revilla et al. investigated several different extraction methods, and concluded that a three step extraction with methanol would give the most reliable results since long time extractions with acidified solvents hydrolyzed malvidin-3-*O*-acetylglucoside to malvidin-3-*O*-glucoside (Revilla et al. 1998). The method of Kennedy et al. was first developed for analysis of grape seed phenols (Kennedy et al. 2000), but was later used for the analysis of grape skins as well (Des Gachons and Kennedy 2003). Recently, Mane et al. optimized the solvent

composition, extraction time, and solvent to solid ratio on the extraction of phenols from grape skin, seed, and pulp (Mane et al. 2007). A major drawback of all these methods is the long extraction time and the manual work required, which complicate automation of the methods for routine analysis

2.3.3 Relations between grape phenols, wine phenols and wine color

It is generally accepted that the chemical composition of grapes directly influences the composition of the wine and thereby the wine characteristic. However, only a few studies have investigated the relation between the phenolic composition of grapes and wines.

The total anthocyanins levels in red grapes have been shown to correlate well (27 samples, $R^2 = 0.82$) to the color intensity of wines, having bisulfite bleaching and pH effects removed (Iland 1987). Since the extent of anthocyanin extraction can vary greatly during winemaking, it was concluded that this method should be regarded as an estimate of the color potential of the grapes under optimal extraction conditions. The relation between grape color and both wine color, wine flavor, and overall wine quality have also been reported for wines made from Shiraz grapes (Francis et al. 2008, Gishen et al. 2002).

It has also been found, that the extraction of anthocyanins from grapes varies according to the maturity of the grapes and the state of the skin cells (Ribéreau-Gayon et al. 2006). The extraction method of Saint-Cricq De Gaulejac et al. allows the estimation of the extractability of grape anthocyanins (Saint-Cricq De Gaulejac et al. 1998). The method was developed due to an observation, that grapes produced from grapes with high anthocyanin content not necessarily lead to a highly colored wine (Ribéreau-Gayon et al. 2006). Good correlation coefficients ($r > 0.95$) between the wine color intensity and grape anthocyanins have been found using the extractability assay (Gonzalez-Neves et al. 2004). In this study, anthocyanins extracted at both pH 1 and pH 3.2 correlated equally well to the color intensity, indicating no clear advantage of using the extractability index.

A recent study found significant correlation coefficients ($r = 0.64$ and $r = 0.69$) between the color intensity of the red wines and grape anthocyanins extracted at pH 1 and pH 3.6 respectively (Romero-Cascales et al. 2005). However, color measurements for pH normalized wines using Boulton's color analysis (Levengood and Boulton 2004) correlated much better with anthocyanins extracted at pH 3.6 ($r = 0.86$) than anthocyanins extracted at pH 1 ($r = 0.25$) (Romero-Cascales et al. 2005). Even though these correlations were only based on five samples, it indicates that anthocyanin extractability may affect the relationship between grape and wine samples.

2.4 Measurement of phenols by spectroscopy

2.4.1 FT-MIR spectroscopy in the wine industry

Many methods for compositional analyses of foods are time consuming and are thus difficult to implement in the routine analysis of food products and raw materials. Vibrational spectroscopy allows samples to be analyzed much faster and has many applications in the food industry, e.g. analysis of dairy products and wine (Andersen et al. 2002).

Most organic compounds absorb infrared radiation due to vibrations in the chemical bonds. The absorption frequency depends on the bond type and surroundings of the vibrating chemical bond and the infrared spectra of different molecules have different spectral characteristics. The infrared spectra thus contain information of the chemical composition of the analyzed sample.

Many compounds in wine and grape juice will absorb in the same regions of the infrared spectra and interfere with the signals of the analyte of interest. In addition, the absorptions of the major wine or grape components (in particular water, ethanol, and sugars) will dominate the infrared spectra (Patz et al. 2004). To overcome these issues, multivariate calibration techniques are used to find the relevant information in the spectra.

Multivariate regression techniques can be used to find good regression models (**y** = **Xb** + **f**), which predicts the desired property (**y**) from the multivariate data (**X**) and regression coefficients (**b**), by minimizing the residual error (**f**). Partial least squares (PLS) regression is a highly used technique for developing multivariate regression models. Briefly, PLS regression is a bilinear modeling method which projects the many variables in **X** into a set of fewer latent variables optimized to describe as much information in **y** as possible (Esbensen 2000a). Although PLS can handle noisy data, it may be beneficial for the predictive ability of the PLS models to select the most informative variables (Leardi 2000, Norgaard et al. 2000). Developing multivariate calibration models includes a risk of overfitting the data. Thus the performance of the calibration model should be validated with an independent test set, which has not been included in the calibration development (Esbensen 2000b).

The first purpose-built Fourier-transform mid infrared (FT-MIR) spectrometer for wine analysis (Winescan FT120, Foss, Hillerød, Denmark) was marketed in 1998 (Andersen et al. 2002). This instrument allows simultaneously determination of several important components in wine, fermenting must, or grape juice samples in less than 90 seconds. Good performance of FT-MIR was recently reported for several components (e.g. alcohol, sugars, glycerol, total phenols, total acids) in wines (Patz et al. 2004). Some components (e.g. total SO_2, lactic acid, and citric acid) in very low concentrations were not accurately determined, and it was concluded that FT-MIR was not suitable for components under 0.2 g/L and that the concentrations lower than 1 g/L only gave semi-quantitative results.

2.4.2 Rapid measurement of phenols

The measurement of phenols in grapes and wine by different spectroscopic techniques, in particular MIR, NIR/VIS and UV/VIS, has been reported. Table 4 gives an overview of reported results of determination of phenolic composition in grapes and wines by spectroscopic methods.

The overview shows, that the levels of total phenols and anthocyanins in wine are best determined from FT-MIR spectra of the wines or from UV/VIS spectra of diluted wine samples, while NIR/VIS spectra of wines gives poorer results (Patz et al. 2004, Skogerson et al. 2007, Soriano et al. 2007). Skogerson et al. stated that UV/VIS gives better prediction of total phenols than FT-MIR, but from these results the two spectroscopic techniques were concluded to give comparable results. The average

levels of anthocyanins and tannins varied approximately two fold between the studies and reflected that the phenolic levels vary according to the samples and analytical method.

Table 4. Overview of reported results for the measurement of phenols by spectroscopic methods.

Technique / Reference	Sample	Measured parameter	N	Mean	SECV or RMSECV	R^2_{cal}	SEP or RMSEP	R^2_{val}
FT-MIR [1]	Wine	Total phenols	327	570 mg/L [a]	-	-	126	0.96
FT-MIR [2]	Wine	Anthocyanins	323	222 mg/L	34	-	-	0.92
FT-MIR [3]	Wine	Tannins	84	399 mg/L	-	-	54	0.96
FT-MIR [4]	Wine	Tannins	20	-	63	0.96	-	-
NIR/VIS [5]	Wine	Anthocyanins	492	183 mg/L	28	0.92	-	-
NIR/VIS [5]	Wine	PP [b]	358	21.4 mg/L	5.9	0.87	-	-
NIR/VIS [5]	Wine	Tannins	294	318 mg/L	131	0.80	-	-
NIR/VIS [6]	Wine	Total phenols	200	782 mg/L	190	0.77	-	-
NIR/VIS [7]	Grape	Anthocyanins	3135	1.24 mg/g	0.15	0.89	0.16	0.88
NIR/VIS [8]	Grape	Anthocyanins	693	0.73 mg/g	-	-	0.15	0.74
UV/VIS [6]	Wine	Anthocyanins	200	420 mg/L	77	0.89	87	0.88
UV/VIS [6]	Wine	PP [b]	200	1.9 abs	0.53	0.82	0.58	0.76
UV/VIS [6]	Wine	Tannins	200	202 mg/L	56	0.90	66	0.86
UV/VIS [6]	Wine	Total phenols	200	782 mg/L	118	0.91	130	0.88

[a] Median values for this parameter. [b] PP = polymeric pigments. References: [1] (Patz et al. 2004), [2] (Soriano et al. 2007), [3] (Fernandez and Agosin 2007), [4] (Versari et al. 2006), [5] (Cozzolino et al. 2004), [6] (Skogerson et al. 2007), [7] (Janik et al. 2007), [8] (Larrain et al. 2008).

The levels of tannins in young wines and wines during fermentation were more accurately determined by UV/VIS ($R^2 = 0.90$) than NIR/VIS ($R^2 = 0.80$) (Cozzolino et al. 2004, Skogerson et al. 2007). Tannins have also recently been determined in wines with attenuated total reflection FT-MIR spectroscopy, which however required extensive sample purification by solid phase extraction and solvent evaporation (Fernandez and Agosin 2007). FT-MIR spectroscopy of the purified wine samples gave better accuracy than UV/VIS spectroscopy, but also required more sample preparation. In preliminary results, tannins were determined directly from the FT-MIR spectra of wines, but due to the low number of samples (20) and a high number of latent variables (10) for the PLS models the data were most likely highly overfitted (Versari et al. 2006). Wine color attributes measured by Boulton's color assay could also be predicted from the FT-MIR spectra of the wines, but again these results were likely overfitted (Versari et al. 2004, Versari et al. 2006).

NIR/VIS spectroscopy of grape homogenates allows determination of anthocyanins, total soluble solids and pH of red grapes and is currently used in the Australian wine industry for quality assessment of grapes (Gishen et al. 2005, Janik et al. 2007). A clear advantage of this technique is that extraction of the anthocyanins is not necessary and thus simplifies sample preparation. It has also been reported that redness determined from the VIS reflectance of grape must correlates with the anthocyanin content of the measured must (Celotti and Carcereri De Prati 2005). This method was claimed to allow rapid evaluation of the phenolic quality of red grapes, but seemed to be highly dependent on the grape cultivar. An even more simple approach with a handheld instrument uses direct NIR measurements of intact, whole grapes, but also gives poorer prediction of anthocyanins (Larrain et al. 2008).

CHAPTER 3 RESULTS AND DISCUSSION

3.1 Extraction of polyphenols from red grapes (Paper I)

3.1.1 Introduction and scope

In grapes, the majority of the polyphenols are located in the skins and seeds, with only minor amounts present in the juice of the grape. In order to quantify the grape polyphenols, these must first be extracted from the grape tissue, which is typically achieved by solvent extraction. However, one of the major barriers for implementation of analysis of grape polyphenols at wineries is that most extraction methods require long extraction time and tedious multi-step sample preparation.

The objective of this work was to develop an extraction protocol, which would allow a fast and robust quantification of polyphenols in red grapes, with a potential for automation. Furthermore, since the overall objective of this research work was to predict wine characteristics from grape analyses, the extraction protocol should allow the establishment of a relation between the phenolic composition of the corresponding grapes and wines.

3.1.2 Criteria for the extraction protocol

To allow comparison of grape samples having different levels of polyphenols, extractions were benchmarked against the 'total' content of polyphenols in the grapes and expressed as extraction degrees. The benchmark 'total extraction' protocol was based on a slightly modified version of Iland's reported total extraction protocol (Iland et al. 2004), extended with an additional extraction of the residual solids recovered after the first extraction. While more than 95 % of the anthocyanins were extracted in the first extraction step, considerable amounts of total phenols were extracted in the second extraction, accounting for an average of 10.5 % of the total amounts extracted from the grapes (Jensen et al. 2007). Although the Iland extraction extracts a high percentage of the grape phenols, the long extraction time (1 hour) and high dilution factor (10:1 v/w solvent to grape material) are not compliant with rapid spectroscopic measurements.

Optimally, the grape extraction protocol should be rapid, give reproducible results, provide consistent results for different grape cultivars, and provide an extract with a high phenolic concentration. Also the grape extraction protocol should reflect the extraction of polyphenols during winemaking. However, the extraction of polyphenols varies highly according to the winemaking procedures (Sacchi et al. 2005), and therefore it is difficult to design an extraction protocol which is fully mimicking what occurs during winemaking. An alternative approach is to use the total amount of polyphenols in the grapes as an indicator of the phenolic potential of the wines. Using total extractions it has been found that the anthocyanins correlate well with the color of red wines (Iland 1987).

For the development of a fast extraction protocol, we focused on developing an extraction protocol, which would allow extraction of a consistent proportion of the polyphenols from red grapes of different cultivars. Due to low extraction efficiencies of phenols in winemaking it was not considered to be necessary to ensure a complete extraction. Extractions were carried out on frozen grape material, which although

known to increase the extraction of phenols (Jackson 1994b, Sacchi et al. 2005) provided the only feasible starting point for the systematic development of a fast extraction protocol. Using frozen material thus allowed experiments to be conducted independently of the season and allowed experiments to be repeated for the same grape lots.

3.1.3 Factors affecting extraction

The efficiency of phenolic extraction from foods, including various fruits and berries, are known to be affected by several factors, including the solvent type, pH, temperature, number of extraction steps, solvent volume, and particle size (Escribano-Bailon and Santos-Buelga 2003). Methanol or acetone are known to be more efficient extraction solvents for phenols than ethanol (Escribano-Bailon and Santos-Buelga 2003, Yilmaz and Toledo 2006). Nevertheless it was decided to use ethanol for the extractions due to its natural role in winemaking and its less hazardous properties as compared to methanol or acetone.

As a starting point for the development of a fast extraction protocol, we focused on investigating the impact of selected factors on the extraction of polyphenols from homogenates of different red grape cultivars. The effects of the factors were initially estimated in statistically designed experiments, using the extraction degrees of total phenols and anthocyanins as responses.

Effect of solvent to solid ratio

A low solvent to solid ratio in the extractions was desirable, since it would produce a high concentration of polyphenols in the grape extract and minimize the solvent consumption. On the other hand, the extraction degree could be negatively affected by a too low solvent to solid ratio, as reported for grape pomace (Pinelo et al. 2005). Based on a preliminary trial it was found, that a solvent:homogenate ratio of 1:1 v/w gave similar levels of phenolic extraction from grape homogenates as higher solvent: homogenate ratios (Figure 14). Hence this solvent:homogenate ratio of 1:1 was considered appropriate for developing a fast extraction protocol.

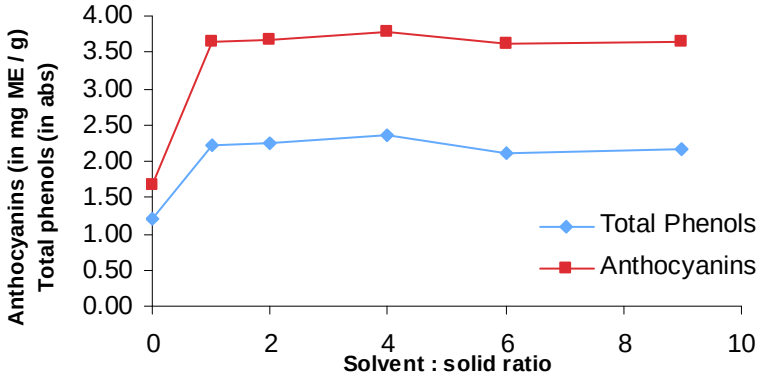


Figure 14. Extraction yields (per mass of grape) of polyphenols from red grape homogenates (Alicante) by extraction for 30 minutes with 50 % ethanol (pH 2) at different solvent:homogenate ratios (0, 1, 2, 4, 6 and 9 v/w). (Jensen, unpublished data).

Extraction temperature and solvent concentrations of ethanol and HCl

The effect of extraction temperature (20, 40 and 60 ºC) and solvent concentrations of ethanol (0, 25 and 50 % v/v) and hydrochloric acid (0 and 0.1 M) were tested in full factorial experimental designs with three center points using the extraction degrees of

total phenols and anthocyanins as responses. As a starting point the extraction time was fixed at 30 minutes, including a significant time for heating up the samples (around 10 minutes). This extraction time was decided from a screening study, where no significant differences between 15 and 30 minutes extraction and a significant negative effect of very long extraction time (150 minutes) was observed. From screening experiments, solvent levels of HCl between 0 and 0.1 M were considered the most appropriate range with a positive effect on the extraction yield (data not shown).

The experimental plan was repeated for nine different samples covering eight different grape cultivars. For each factorial combination the mean value and the relative standard deviation of the extraction degree were calculated (Jensen et al. 2007). The relative standard deviations of the extraction degree indicated how consistent the extraction degrees were across different grape cultivars, and were found to be negatively related with the mean extraction degree (Figure 15). This showed that a high extraction degree was desirable for the development of a robust extraction method, and that the relative standard deviations between different samples in practice could be as low as ~5 % for total phenols and ~2 % for anthocyanins.

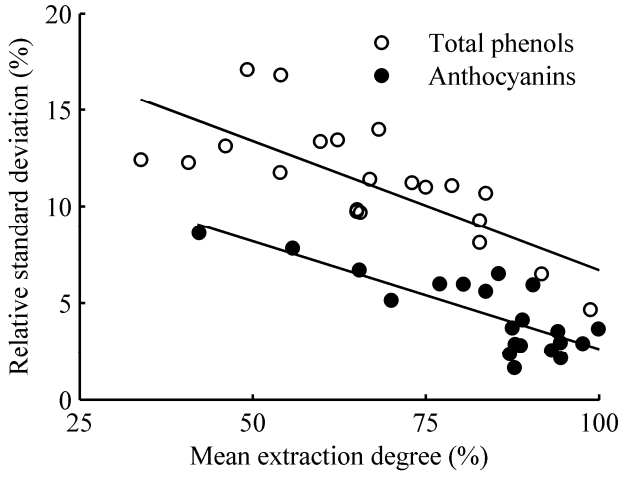


Figure 15. Correlation between mean extraction degrees (%) and relative standard deviation between the nine samples (%) for the extraction of total phenols and anthocyanins (Jensen et al. 2007).

The extraction degree of total phenols and anthocyanins was found to be highly influenced by both the extraction temperature and the solvent concentrations of ethanol and HCl (Figure 16).

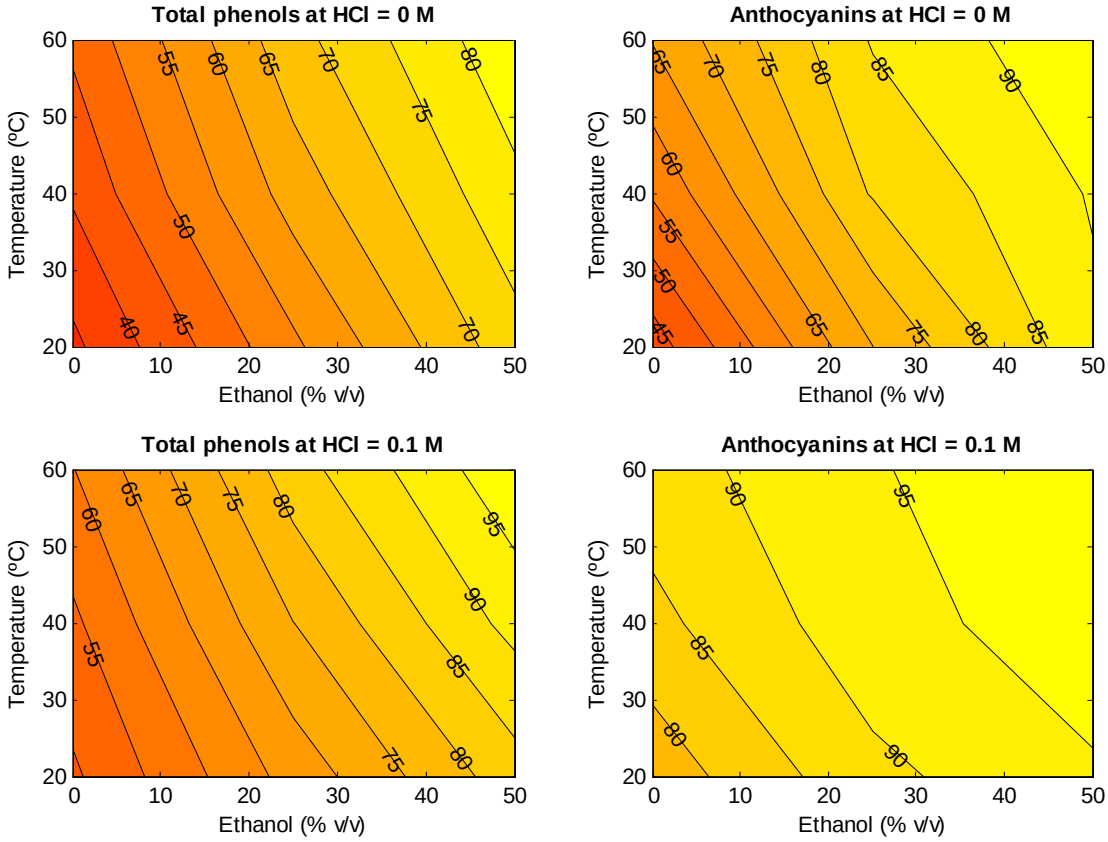


Figure 16. Contour plots of the effect of ethanol and temperature on the extraction degrees of total phenols and anthocyanins at both 0 and 0.1 M HCl (Jensen, unpublished data). Numbers on the contour lines specify the extraction degrees.

The effects of the factors were estimated by fitting the responses (y_i) to a linear model of the three factors (x_1, x_2, and x_3), accounting for both main and interaction effects:

$$y_i = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \varepsilon_i$$

From the β estimates, it was concluded that the concentration of ethanol had the highest impact on the extraction of both total phenols and anthocyanins (Table 5). The extraction temperature and solvent concentration of HCl also had a substantial impact on the extraction degree. No significant interaction effects between the three factors were observed for the extraction of total phenols, while ethanol showed negative interaction effects with the two other factors for the extraction of anthocyanins. The negative interaction effects observed for anthocyanins either indicated anthocyanin degradation, or more likely just reflected the inability of the statistical model to account for the extraction degrees of anthocyanins approaching a complete extraction at the high factor levels.

Table 5. Effect tests and estimated model parameters for the mean extraction degrees (%) of total phenols and anthocyanins in the factorial designed experiments (Jensen et al. 2007).

Term	Total phenols (model fit: $R^2 = 0.99$)		Anthocyanins (model fit: $R^2 = 0.96$)	
	Prob > F [a]	β estimate [b]	Prob > F [a]	β estimate [b]
Intercept	<.0001	66.49	<.0001	83.40
EtOH	<.0001	18.75	<.0001	12.80
HCl	<.0001	6.69	<.0001	8.00
Temp	<.0001	6.63	<.0001	5.48
EtOH·HCl	0.446	-0.37	<.0001	-5.54
EtOH·Temp	0.425	0.48	0.032	-2.92
HCl·Temp	0.367	0.45	0.093	-1.80

[a] Prob > F describes the probability that a term does not have a significant effect.
[b] The β estimates of the linear model using mean centered factor levels scaled between -1 and +1.

From these findings it was concluded that high extraction degrees of both total phenols and anthocyanins were obtained by extraction at elevated temperatures using a solvent with 50 % ethanol and 0.1 M HCl. Even though an extraction temperature of 60 ºC caused the highest extraction degrees, it was decided to only apply an extraction temperature of 40 ºC in the extraction protocol, to allow a substantial reduction of the time needed to heat up the sample under extraction, as described in the following section.

Extraction time

In order to reduce the extraction time of the extraction method, further optimization potential was tested. First of all, the time for heating the sample to 40 ºC was eliminated by preheating the extraction solvent prior to extraction. Secondly, the extraction degrees of reduced extraction times were compared for eight different grape cultivars. These results showed that it was possible to reduce the solvent contact time from 15 minutes to only 5 minutes with only very small decreases of less than 2 % in the extraction degrees of both total phenols and anthocyanins, negligible differences in the repeatability (rel SD ~ 1%) of the duplicate measurements, and negligible changes in the extraction degree consistencies (rel SD ~ 5%) between the samples (Jensen et al. 2007). The extraction degrees after 5 minutes solvent contact time were 93.5 % for total phenols and 98.9 % for anthocyanins, but unfortunately slightly overestimated due to sample turbidities, caused by insufficient sample clarification (data not shown).

3.1.4 Final protocol

To avoid the potential risk of degrading some polyphenols by a long exposure to the acidified solvent, and because some analytical methods are incompatible with highly acidic samples (in particular the Winescan ™), a post extraction step to neutralize the added HCl (with NaOH) was included in the final extraction protocol. In addition a better sample clarification was obtained, by including a filtration step and a higher centrifugation speed for the sample preparation post extraction. Although it has been reported, that acid treatments can cause degradation of some phenolic compounds (Revilla et al. 1998) the short acid treatment used in the final protocol did not lead to any detectable phenolic degradation (Jensen et al. 2007).

The final extraction protocol was evaluated for eight different grape cultivars (Table 6). The average extraction degrees of 81.8 % for total phenols and 91.5 % for anthocyanins showed that the majority of the polyphenols were extracted. The relative standard deviations of the extraction degrees across the different cultivars were 6.0 %

for total phenols and 3.8 % for anthocyanins. The repeatability for the complete extraction protocol, including: sampling of grapes, homogenization of grapes, the actual extraction and neutralization, work-up of the sample and the measurements were estimated from three samples analyzed in triplicates to be 5 % for total phenols and 3 % for anthocyanins (Jensen et al. 2008a).

Table 6. Results of total extraction and fast extraction followed by sample neutralization of eight red grape cultivars (Jensen et al. 2007).

Cultivar	Total phenols (0.01 abs/g) [a]			Anthocyanins (mg/g) [b]		
	Total extraction (N=3)	Fast extraction (N=1)	Extraction degree (%)	Total extraction (N=3)	Fast extraction (N=1)	Extraction degree (%)
Alicante 2	2.16	1.92	88.9%	2.81	2.58	91.8%
Merlot 1	2.22	1.92	86.4%	1.91	1.89	98.9%
Syrah 2	1.77	1.44	81.6%	1.79	1.56	87.1%
Cinsault 1	1.03	0.84	81.8%	0.68	0.63	91.8%
Grenache Noir 2	1.28	1.04	81.2%	0.89	0.82	92.1%
Carignan 2	1.43	1.17	81.7%	1.52	1.39	91.3%
Cab. Sauv. 1	1.86	1.51	80.9%	1.56	1.41	90.4%
Mourvedre 1	1.78	1.28	72.2%	1.40	1.24	88.6%
Mean [c]	1.69	1.39	81.8%	1.57	1.44	91.5%
rel SD [c]	24.7%	28.0%	6.0%	41.5%	42.3%	3.8%

[a] Total phenols are expressed as 0.01 absorbance units per g grape
[b] Anthocyanins are expressed as mg malvidin-3-glucoside equivalents per g grape.
[c] Mean and relative standard deviations across the eight cultivars.

3.1.5 Discussion, conclusion and future perspectives

From the investigation of the influence of selected factors on the extraction degree of polyphenols from frozen grapes, it was possible to determine relevant conditions for a rapid extraction protocol. The developed rapid extraction protocol extracted a high percentage of the total phenols (81.8 %) and anthocyanins (91.5 %) from the grapes, with a short solvent contact time. The shortening of the extraction time is a very clear advantage compared to other reported extraction methods, which typical employs extraction times between 1 and 24 hours (cf. section 2.3.2).

Although at present the routine of the extraction protocol, in particular the clarification step is quite labor intensive, the reduction of the solvent contact time is a crucial step for the development of an eventual automation of the extraction process. Further development of the optimal extraction conditions should focus on employing extraction equipment that would allow the extraction method to be less labor intensive and give a faster overall protocol. Some aspects of this could include the following steps:

- Combining the homogenization and extraction step
- Optimizing the extraction for an even shorter extraction time
- Implementation of a fast and easy sample work-up, which is compliant with FT-MIR measurements (e.g. by cross flow filtration)

The final extraction protocol defined in this study will therefore form the basis for an investigation of how the results can be used for evaluating the winemaking potential of the grapes. These results are discussed in section 3.2 and 3.3.

3.2 Relation between grape and wine polyphenols (Paper III)

3.2.1 Introduction and scope

The objective of this work was to investigate and establish a relation between the phenolic compositions of red grapes with those found in corresponding young red wines. Polyphenols are slowly extracted from the grapes during the primary wine fermentation with a gradually increasing ethanol concentration – and the phenolics in wines then obviously stem from the grapes. However, even with prolonged extraction time, the extraction of polyphenols from grapes during red wine making rarely exceeds 50 % of the total grape phenolic content (Haslam 2005). Also physiological and chemical changes, such as degradation, polymerization, derivatization, adsorption, and precipitation of polyphenols will occur during winemaking and impact the phenolic composition of the finished wines (Cheynier et al. 2006, Fulcrand et al. 2006, Mazauric and Salmon 2006). In addition, winemaking conditions, such as fermentation temperature, maceration time, must freezing, and enzyme additions are known to affect the extraction of polyphenols during winemaking (Sacchi et al. 2005). All this complicates the establishment of a relation between grape and wine phenolic composition, which is an important prerequisite to allow prediction of wine quality parameters from grape phenolic measurements. Nevertheless, a fundamental hypothesis of the present PhD thesis was that the phenols present in the grapes have a critical significance on the phenolic composition in young wines, and that it may be possible to predict phenolic composition of wines from the phenolic composition of grapes.

3.2.2 Polyphenols in grapes and wines

Production of wines

Wines were produced by experimental microvinification (250 g grapes per wine fermentation), with a long maceration and fermentation time of 14 days at 25 ºC. The long maceration time was used to ensure complete fermentations, minimize effects from variations in fermentation kinetics, and for the wine making conditions to reflect the potential of extraction from the grapes. The phenolic composition of the grapes was determined via use of the developed fast extraction method, by which a high extraction of grape polyphenols was obtained with a short solvent contact time (Jensen et al. 2007). Considerable variation in the sugar levels in the grapes and thereby the ethanol levels in the wines were observed (Table 7).

Table 7. Grape sugar content and wine alcohol levels for the studied cultivars. ANOVA showed significant differences ($p < 0.05$) between cultivars for both grape sugar and wine alcohol levels (Jensen et al. 2008a).

Cultivar	N	Grape sugar (Brix)			Wine alcohol (% v/v)		
		Range	Mean	SD	Range	Mean	SD
All samples	55	18.5 - 25.6	22.8	1.8	10.4 - 15.4	13.6	1.2
Alicante	4	18.6 - 21.1	19.6	1.1	10.4 - 12.9	11.5	1.0
Cabernet Sauvignon	4	21.3 - 24.9	22.8	1.8	12.5 - 14.4	13.3	0.9
Carignan	4	20.4 - 22.3	21.4	0.8	12.0 - 13.2	12.8	0.5
Cinsault	4	18.5 - 24.6	21.8	2.7	10.5 - 14.8	13.1	1.8
Grenache	4	20.7 - 23.3	22.5	1.2	12.3 - 14.2	13.6	0.9
Merlot	27	21.2 - 25.6	23.7	1.1	12.1 - 15.4	14.2	0.8
Mourvedre	4	19.8 - 22.2	21.3	1.1	11.9 - 13.3	12.9	0.7
Syrah	4	20.9 - 25.2	23.3	1.8	11.8 - 15.0	13.5	1.4

Differences between the eight cultivars in the study, with relatively low sugar levels in some Alicante, Mourvedre, and Cinsault samples and high sugar levels in Merlot, Syrah, and Cabernet Sauvignon were also evident (Table 7).

Phenolic composition of grapes and wines

The phenolic composition of the grapes and wines were measured by spectroscopic measurements, HPLC, and an assay for the measurement of tannins, and monomeric and polymeric pigments. Hereby both total phenolic levels and more detailed levels of the different phenolic classes were measured (Table 8). Relative standard deviations of 23 – 77 % showed that the phenolic levels of the samples varied substantially. The lowest variations between the samples were observed for the levels of total phenols in grapes (23 %) and wines (27 %), while the levels of most individual classes varied much more across all the samples with relative standard deviations as high as 79 % (Table 8). The observed high extent of variation in the phenolic composition of the grapes was desirable for ensuring robust models, and was obtained by collecting grapes, expected to vary in the phenolic composition.

Table 8. Phenolic composition of grape extracts and red wines produced in parallel from 55 different grape samples covering eight cultivars (Jensen et al. 2008a).

Phenolic compound [a]	Grape composition (per kg grape)			Wine composition (per kg grape)		
	Range	Mean	relSD	Range	Mean	relSD
Total phenols (0.01 abs)	795 - 2356	1518	23%	300 - 1110	665	27%
Anth-spec (mg ME/kg)	533 - 3095	1258	43%	177 - 1173	518	38%
MP (abs)	1.5 - 10.1	3.4	51%	0.74 - 6.5	2.2	46%
SPP (abs)	0.24 - 1.0	0.45	37%	0.23 - 1.4	0.67	38%
LPP (abs)	0.18 - 1.3	0.53	50%	0.09 - 0.84	0.28	50%
Tannins (mg CE/kg)	1129 - 4260	2662	28%	370 - 1479	860	38%
PP (abs)	0.45 - 2.0	0.98	38%	0.32 - 2.2	0.95	40%
Gallic acid (mg/kg)	0.72 - 7.5	3.4	52%	6.1 - 63	23	42%
(+)-catechin (mg/kg)	33 - 271	127	47%	23 - 197	94	49%
(-)-epicatechin (mg/kg)	18 - 236	114	51%	7.1 - 171	77	57%
Hydroxycin. (mg CFAE/kg)	7.2 - 160	54	57%	4.0 - 64	12	79%
Flavonols (mg RUE/kg)	96 - 459	254	34%	28 - 184	86	45%
Anth-HPLC (mg ME/kg)	521 - 3007	1267	42%	136 - 949	392	39%

[a] The abbreviations for the phenolic compounds are: Anthocyanins by spectroscopy (Anth-spec), Monomeric pigments (MP), Small polymeric pigments (SPP), Large polymeric pigments (LPP), Polymeric pigments (PP), and Anthocyanins determined by HPLC (Anth-HPLC).

In general, grape levels of total phenols (between 795 and 2356 abs) and anthocyanins determined by spectroscopy (between 533 and 3095 mg ME/kg) were in agreement with reported levels (Cynkar et al. 2004). The tannin content of the wines were between 370 and 1479 mg CE/kg grapes (equivalent to 430-1718 mg CE/L) and reflected medium to high values of the typical tannin levels reported for red wine in the literature (Fernandez and Agosin 2007, Heredia et al. 2006, Kennedy et al. 2006a, Skogerson et al. 2007). The high tannin levels found in some of the wines indicated that a high extraction from the grapes was obtained, likely as a result of the long maceration time. The anthocyanin content as determined by HPLC of the wines was between 136 and 949 mg ME/kg grapes (equivalent to 158-1102 mg ME/L), and showed that the freshly fermented wines contained higher levels of anthocyanins than typical levels in commercial red wines (De Beer et al. 2004). This is in good accordance with the known conversion of anthocyanins to more stable polymeric

pigments (Somers 1971). This was also reflected in the levels of polymeric pigments (PP), which were approximately two times lower than the levels reported in aged wines (Harbertson et al. 2003).

Sample characterization by PCA

Principal component analysis (PCA) was used to identify the most profound variations in the phenolic composition of the grape samples. The first principal component explained 51 % of the variation and was associated with anthocyanins, polymeric pigments, total phenols, flavonols, and hydroxycinnamates (Figure 17). The second principal component was associated with the catechins, gallic acid and tannins. The PCA revealed some differences between the cultivars, for instance high tannin levels in Merlot and Cabernet Sauvignon, high anthocyanin levels in Alicante, and low overall phenolic levels in Carignan, Grenache, and Cinsault grapes. Overlaps in the PCA plot between several cultivars did not allow a clear differentiation between the grape cultivars using only two principal components. Nevertheless, the differences between grape cultivars were larger than differences within the grape cultivars. The differences between some cultivars were more profound for some cultivars (e.g. Cabernet Sauvignon) than others (e.g. Cinsault).

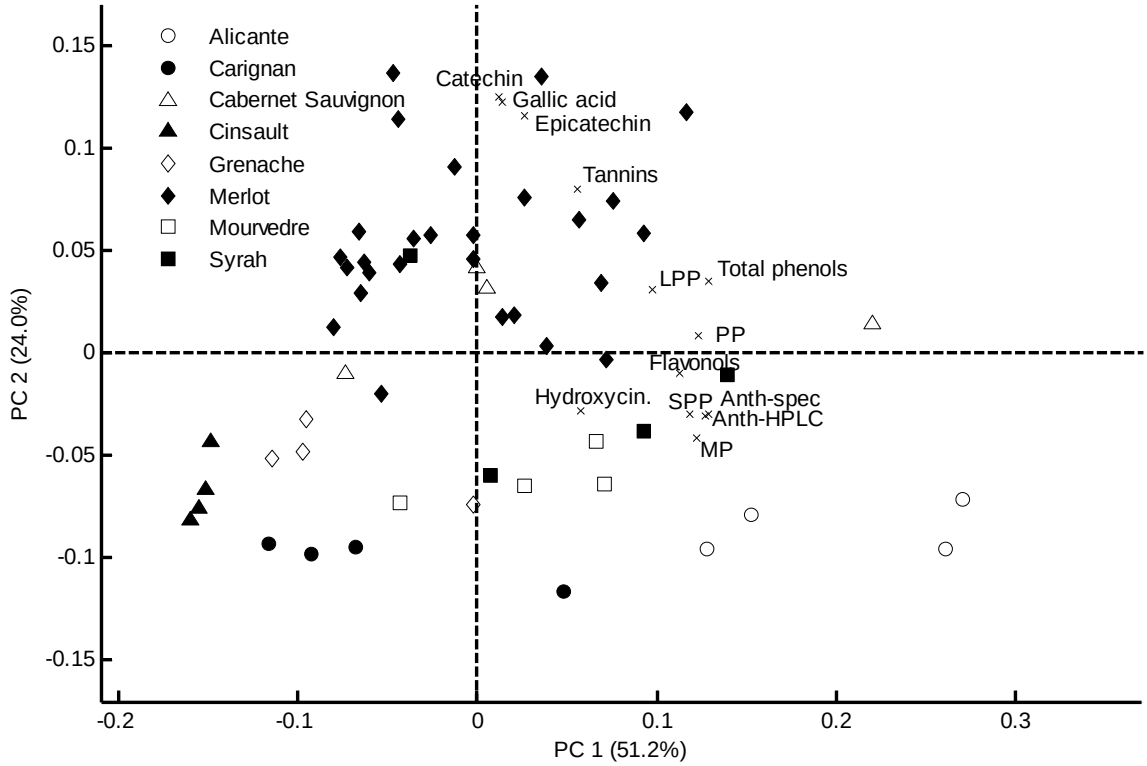


Figure 17. Bi-plot of scores and loadings from the PCA of the phenolic composition of the grapes (Jensen et al. 2008a).

3.2.3 Relation between grape and wine polyphenols

Ratios of wine/grape phenols

The ratio between the phenolics in wines and grapes (Table 9) described how large a proportion of the grape phenols that was recovered in the wine. The average ratio of 0.44 for total phenols was in good accordance with the general observation, that extraction of phenols from grapes rarely exceeds 50 % during winemaking (Haslam 2005). However large differences were observed between the different phenolic

compounds. An average ratio for tannins of 0.31 showed that only a small proportion of the grape tannins were recovered in the wine, which could be a consequence of the known slow extraction of tannins (Sacchi et al. 2005). The low average ratio of 0.31 for anthocyanins determined by HPLC (Table 9) reflected that anthocyanin concentrations usually peak early in the maceration process after which the concentration drops (Nagel and Wulf 1979). The high average ratio of 7.9 for gallic acid showed that gallic acid was released during winemaking in quantities exceeding the small initial levels present in the grapes, which was likely due to a release of gallic acid from ester hydrolysis during winemaking (Oszmianski et al. 1986). The relative standard deviation of the ratios between grape and wine phenolics indicated how consistent the proportions of grape phenols were recovered in the wine. Total phenols, anthocyanins, (+)-catechin, and monomeric pigments were recovered most consistently, while the other phenolic classes were less consistently recovered, in particular hydroxycinnamates, gallic acid, and large polymeric pigments (Table 9).

Table 9. Ratios, direct relation, and multivariate relation between grape and wine phenolics for all 55 samples (Jensen et al. 2008a).

Phenolic compound	Ratios [a]	Direct relation [b]		Multivariate relation [c]		
	Wine / grape	r [e]	RMSECV [g]	LV [d]	r [e]	RMSECV [g]
Total phenols (0.01 abs)	0.44 (±13%)	0.880	86 (13%)	2	0.910	75 (11%)
Anth-spec (mg ME/kg)	0.42 (±12%)	0.941	67 (13%)	5	0.932	61 (12%)
MP (abs)	0.66 (±17%)	0.897	0.45 (20%)	6	0.896	0.39 (18%)
SPP (abs)	1.5 (±24%)	0.801	0.15 (22%)	5	0.916	0.09 (13%)
LPP (abs)	0.57 (±43%)	0.384	0.13 (46%)	1	0.798	0.08 (30%)
Tannins (mg CE/kg)	0.32 (±27%)	0.653	244 (28%)	3	0.754	205 (24%)
PP (abs)	0.98 (±24%)	0.755	0.25 (26%)	5	0.910	0.14 (15%)
Gallic acid (mg/kg)	7.9 (±47%)	0.608	7.7 (33%)	2	0.671	7.2 (31%)
(+)-catechin (mg/kg)	0.75 (±16%)	0.954	14 (15%)	8	0.912	15 (16%)
(-)-epicatechin (mg/kg)	0.66 (±22%)	0.948	14 (18%)	5	0.888	13 (17%)
Hydroxycin. (mg CFAE/kg)	0.25 (±79%)	0.735	6.3 (53%)	6	0.230	6.2 (52%)
Flavonols (mg RUE/kg)	0.34 (±28%)	0.757	25 (29%)	7	0.518	23 (27%)
Anth-HPLC (mg ME/kg)	0.31 (±12%)	0.934	54 (14%)	6	0.911	53 (13%)

[a] Average ratios (± relative standard deviation) between the levels of wine and grape polyphenols. [b] The direct relation between grape and wine was evaluated using a one factor PLS model with full cross validation. [c] Multivariate relation was evaluated from all 13 phenolic compounds of grapes (autoscaled data) using PLS model with full cross validation. [d] LV is the number of latent variables used for the PLS model. [e] r is the correlation coefficient between the predicted and measured phenolic concentration. [g] RMSECV is the cross validated root mean square error of prediction, with the % of the mean given in the brackets.

Direct relation between grape and wine phenols

The direct relations between the specific grape and wine phenolic classes were investigated to evaluate the feasibility of estimating wine phenolic levels from grape measurements. In addition, because phenolic composition is known to change during winemaking, in particular formation of polymeric pigments (Somers 1971), it was attempted to estimate the phenol composition of wines from the detailed phenolic composition of grapes using partial least squares regression. The best direct relations between grapes and wines were found for total phenols, anthocyanins, monomeric pigments, (+)-catechin, and (-)-epicatechin, while a direct relation for the other phenolic compounds was less evident (Table 9). The direct relation (without cross validation) between grape and wine levels of anthocyanins (Figure 18A) were much better than for tannins (Figure 18 B).

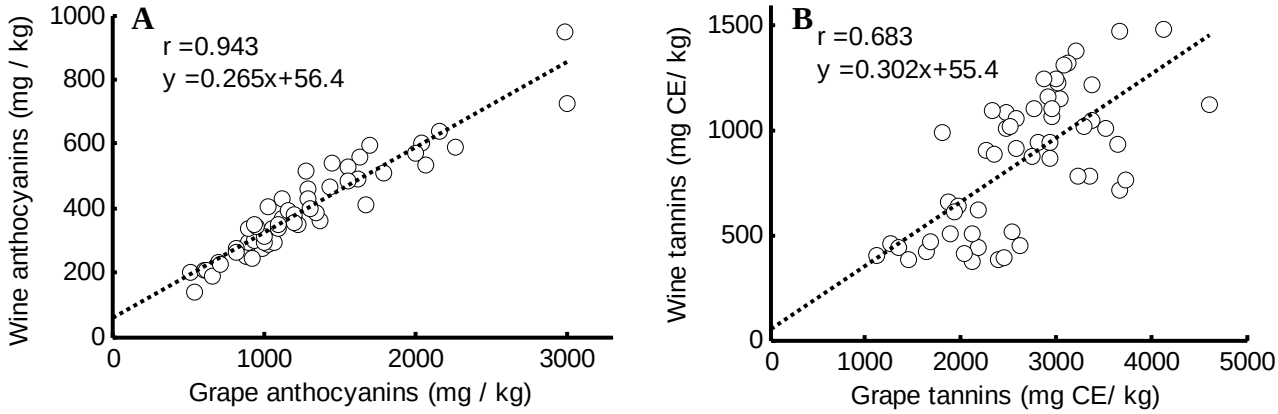


Figure 18. Direct relations between grape and wine levels of A) anthocyanins and B) tannins for the 55 samples (Jensen, unpublished data).

Multivariate relation between grape and wine phenols

In general, only small improvements were obtained for the multivariate relations as compared to the direct relations. The largest improvements using a multivariate approach were found for the polymeric pigments (both PP, LPP, and SPP), giving ~ 40 % lower RMSECV values (Table 9). This was ascribed to the known formation of polymeric pigments during winemaking, e.g. by aldehyde-mediated condensations between anthocyanins and flavonols (Fulcrand et al. 2006). This was also supported by the finding, that the correlation coefficients between grape anthocyanins and wine polymeric pigments ($r_{PP} = 0.87$, $r_{SPP} = 0.87$, and $r_{LPP} = 0.80$) were higher than the correlation coefficients between grape and wine polymeric pigments ($r_{PP} = 0.78$, $r_{SPP} = 0.82$, and $r_{LPP} = 0.48$), showing that wine polymeric pigments correlated well with grape anthocyanins (Jensen et al. 2008a).

Direct relations for Merlot samples

The direct relations between grape and wine phenols were also investigated for only the Merlot samples, to evaluate if better models could be made for a specific grape cultivar (Table 10).

Table 10. Direct relation between grape and wine phenolics for the 27 Merlot samples (Jensen et al. 2008a)

Phenolic compound	Direct relation [a]	
	r [b]	RMSECV [c]
Total phenols (0.01 abs)	0.834	54 (7%)
Anth-spec (mg ME/kg)	0.920	46 (9%)
MP (abs)	0.906	0.23 (10%)
SPP (abs)	0.617	0.13 (18%)
LPP (abs)	-0.135	0.11 (35%)
Tannins (mg CE/kg)	0.641	130 (12%)
PP (abs)	0.405	0.22 (22%)
Gallic acid (mg/kg)	0.155	9.3 (32%)
(+)-catechin (mg/kg)	0.845	13 (10%)
(-)-epicatechin (mg/kg)	0.865	12 (11%)
Hydroxycin. (mg CFAE/kg)	0.521	3.5 (34%)
Flavonols (mg RUE/kg)	0.891	16 (17%)
Anth-HPLC (mg ME/kg)	0.888	44 (12%)

[a] The direct relation between grape and wine was evaluated using a one factor PLS model with full cross validation. [b] r is the correlation coefficient between the predicted and measured phenolic concentration. [c] RMSECV is the cross validated root mean square error of prediction, with the % of the mean given in the brackets .

Including only the Merlot it was found that the RMSECV values of the direct relation between grape and wine phenols improved for all phenols (cf. Table 9 and Table 10), except for gallic acid which is likely due to the observation that the gallic acid found in grapes only account for a very small proportion of the gallic acid levels found in wine. While the RMSECV values were improved, the correlation coefficients were mainly lower for only the Merlot samples than all samples, due to a smaller variation in the phenolic composition. Interestingly, improvements were observed for tannins, where the RMSECV decreased from 244 to 130 mg CE/kg grapes, which might indicate that cultivar specific models could establish better relations between grape and wine phenols, in particular for tannins.

3.2.4 Discussion, conclusion and future perspectives

Using experimental winemaking conditions the phenolic composition of grape extracts and wine phenolics were investigated. The winemaking conditions used in this study involved a long maceration time and produced wine with a normal to high level of tannins, which indicated that the extraction from the grapes was higher than under typical commercial conditions. The proportion of wine phenols recovered from the grapes varied from 0.25 to 7.9, which likely both reflected incomplete extractions of tannins, chemical reaction of the anthocyanins, and release of gallic acid during the wine making process.

Good direct relationships between grape and wine composition were found for anthocyanins, total phenols, (+)-catechin, and (-)-epicatechin, while direct relations between the other phenolic compounds were less evident. The direct relation between grape and wine tannins was poor, likely due to that the conditions used for grape extraction were not representative for winemaking. In particular, the thorough crushing of seeds could lead to unrepresentative extractions of tannins.

Using a multivariate approach it was possible to improve the relation between grape and wine phenols, especially for the polymeric pigments, which are known to be formed during winemaking. However, the development of multivariate models requires more detailed analysis of the grapes and this approach is therefore not as simple as the use of the direct relation. In addition it was found, that the grape anthocyanins correlated well with the polymeric pigments in wines, which could be the reason for the improved relations for the multivariate models. Hence, it was concluded that the direct relations between the grape and wine phenols were the most appropriate for describing the relation between grape and wine phenols at this point. Considering, that only very young wines were investigated in this study, it can not be concluded if a multivariate approach might be more appropriate if more chemical changes were allowed to occur during aging.

For just the Merlot samples slightly better direct relations between grape and wine phenols were found, in particular for tannins. This was likely a consequence of the grape berries of one single variety cultivar having more similar physiology (e.g. grape and seed sizes) than grapes of different cultivars. Development of more specific models to predict wine compositions within a single cultivar could improve the model performances. However, at this point it seemed that for most parameters the cultivar specific models only gave minor improvements. By balancing the increased effort

required to develop cultivar specific models with the improvements one could expect, it was concluded that a general model covering many cultivars would be preferable.

Future perspectives
Future perspectives should involve an investigation of changes in the phenolic composition during aging, which will be of prime interest for establishing the relation between grape phenols and the phenolic composition of a finished wine. In this study only freshly fermented wines were studied, which provides the starting point for the development of predictive tools for finished wine composition and quality.

3.3 Prediction of wine color attributes (Paper III)

3.3.1 Introduction and scope

The objective of this work was to investigate and establish a relation between the phenolic composition of grapes and selected color attributes of the corresponding young wines. The relation was investigated for the grape extracts and corresponding wines described in Section 3.2.

Wine color is an important part of the quality perception of the wine and the color intensity has been found to correlate with the overall wine quality (Jackson et al. 1978, Somers and Evans 1974). Furthermore wine color is one of the few organoleptic properties, which can be measured objectively and relatively easy.

The color of very young red wines is mainly due to anthocyanins in their highly colored flavylium form. During wine fermentation anthocyanins are extracted from the grape skins into the fermenting must, but will also undergo several chemical changes already during the fermentation (Fulcrand et al. 2006). The formation of polymeric pigments is regarded very important for the color changes as wine ages. In young wines, the wine color will also be affected by non colored phenols, which will enhance wine color by copigmentation (Boulton 2001). Hence wine color is not only related with the levels of anthocyanins, but also levels of other polyphenols including polymeric pigments and copigments. To account for these effects we used a multivariate approach to relate the phenolic composition of red grapes to selected wine color attributes.

3.3.2 Color attributes of red wines

Due to the known effect of pH on the equilibria between the colored and non-colored forms of anthocyanins, all wine color attributes were determined on pH normalized wines (pH = 3.6). Boultons color assay (Levengood and Boulton 2004) was used to estimate the total color intensity at 520 nm of the pH normalized wines with any bleaching effect of bisulfite removed and to estimate the contribution from anthocyanins, copigmentation and polymeric pigments on the wine color (Table 11).

Table 11. Percentages of wine color due to copigmentation, polymeric pigments, and anthocyanins for the 55 young wines (Jensen et al. 2008a).

Wine color due to	range	mean	SD
copigmentation	21-42 %	33 %	4.6 %
polymeric pigments	10-20 %	16 %	2.4 %
anthocyanins	45-61 %	51 %	3.2 %

On average 51 % of wine color was due to anthocyanins, 33 % due to copigmentation, and only 16 % due to polymeric pigments. With standard deviation between 2.4 and 4.6 %, the percentages of the color due to the three factors varied only slightly between the wines.

The variation in the percentages of color due to copigmentation was apparently related to the total levels of anthocyanins in the wines (Figure 19). This relationship indicated that self association of anthocyanins played a major role in the copigmentation of anthocyanins, in accordance with the literature (Boulton 2001).

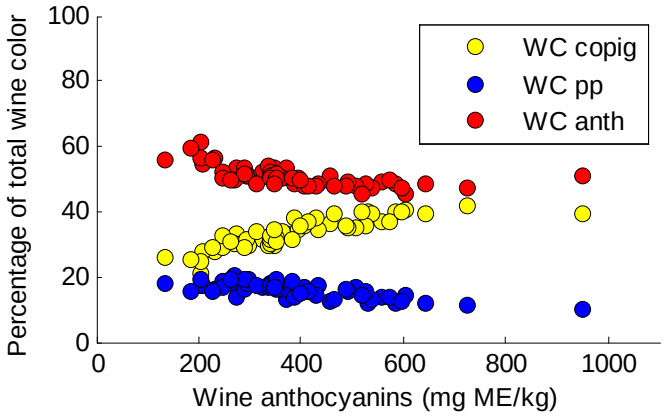


Figure 19. Relation between wine anthocyanin levels and percentage of color due to copigmentation, polymeric pigments, and anthocyanins (Jensen, unpublished data).

In addition, CIELab color values (Perez-Caballero et al. 2003) and color intensity and tonality (Sudraud 1958) were estimated from absorbance readings of the pH normalized wines. Sudraud's color values have traditionally been used to describe the variation of wine color, in particular with regards to monitor the color changes from red to brick red colors during aging. CIELab color values are qualitatively related with the psychological attributes of color (Ayala et al. 1997). The CIELab system describe the color according to the lightness (L*), redness/greenness (a*), blueness/yellowness (b*), chroma (C*), and hue angle (H*). CIELab color values of red wines have been reported using path lengths between 1 and 10 mm, which will highly affect the color values, since 10 mm path lengths will be problematic for dark wines (Ayala et al. 1997), while short path lengths will give low color values. In this study a 2 mm path length was used, since this allowed simplified calculations of the color values from only four characteristic color values (Perez-Caballero et al. 2003).

One wine sample (made from Alicante grapes) with the highest total wine color (32 abs) of all wines, was found to give inaccurate b*, H*, and C* values compared with the values calculated from the official CIE guidelines (Ohta and Robertson 2005c). This indicated that the CIELab calculations as proposed by Perez-Caballero *et al.* (2003) were not valid with extremely high colored samples and this highly colored sample was therefore considered as an outlier and excluded in the following data analysis. The colors described by the CIELab color values showed considerable differences between the samples, and also showed that color calculated for 2 mm path lengths gives light colors (Figure 20). Nevertheless, all wine color attributes were determined with good repeatabilities between 1 and 3 % (Table 12).

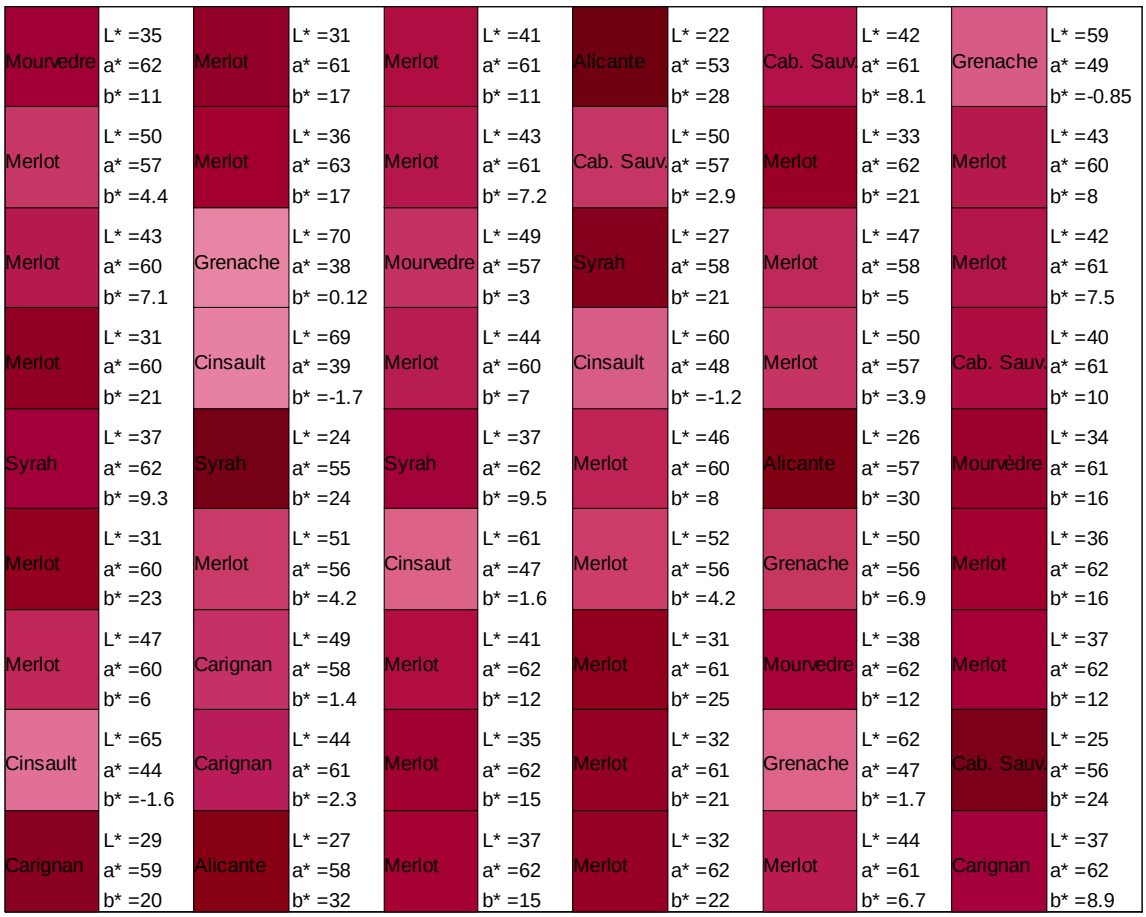


Figure 20. Schematic overview of the colors of the 54 wines as described from the CIELab system (Jensen, unpublished data). The order of samples is randomized.

Table 12. Wine color attributes determined for 54 young wines (Jensen et al. 2008a). Repeatability is determined from triplicate measurements of three samples.

Color attribute	range	mean	SD	Repeatability (%)
Total wine color (WC total)	3.2 - 25	11	4.9	1%
Wine color due to copigmentation (WC copig)	0.8 - 10	3.9	2.1	3%
Wine color due to polymeric pigments (WC PP)	0.50 - 2.9	1.7	0.58	1%
Wine color due to anthocyanins (WC anth)	1.9 - 12	5.5	2.2	1%
L*	22 - 70	42	12	1%
a*	38 - 63	58	5.8	1%
b*	-1.7 - 32	11	8.9	3%
C*	38 - 67	59	6.5	1%
H*	-2.5 - 29	10	8.2	2%
Tonality	0.41 - 0.55	0.47	0.04	1%
Color intensity	0.50 - 3.4	1.6	0.68	1%

The total wine color of the wines was on average higher than the total wine color of 6 months old Cabernet Sauvignon wines (Levengood and Boulton 2004), reflecting that some cultivars in this study was highly colored (in particular Alicante). Compared with Levengood's results, the color due to anthocyanins and polymeric pigments was on average 2.7 times higher and 1.7 times lower respectively, which could reflect that the wines were measured just after fermentation where only minor transformation of anthocyanins into the more stable polymeric pigments would have occurred. The CIELab color values of the wines were in general in agreement with reported values

for wines of varying age (Monagas et al. 2006a), but also included wines that were much darker and with a higher degree of blueness. The color intensity values were also higher than values reported for commercial wines, while the tonality varied very little as opposed to those reported for aged wines (Sudraud 1958).

The relation between the cultivars and the color parameters were analyzed by principal component analysis, by which more than 95 % of the variation was explained using only two principal components (Figure 21). The first principal component explained 84 % of the variation and was associated with variations in b*, H*, color intensity, all Boulton's color parameters, L*, and tonality.

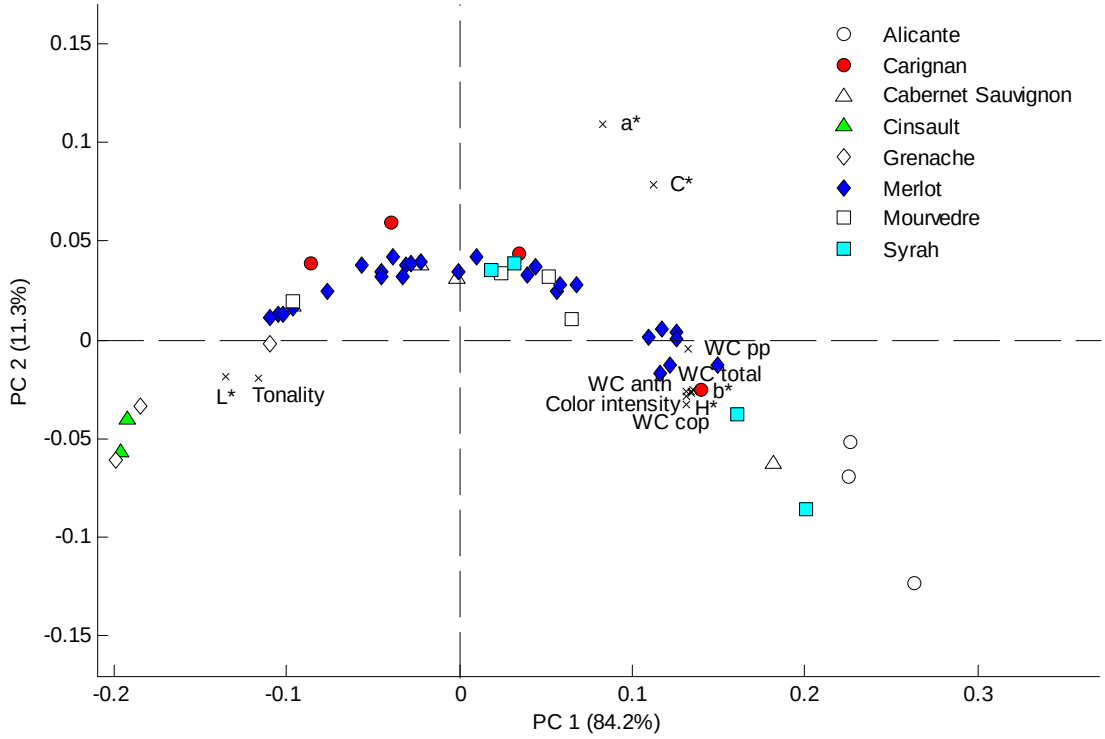


Figure 21. Bi-plot of scores and loadings from the principal component analysis of wine color parameters (auto scaled) for the 54 wines (Jensen, unpublished data).

The second principal component explained 11 % of the variation and was associated with a* and C*. The grouping of the color values in three groups indicated, that most of the color variation was described by either the L*, a*, and b* or the L*, C*, and H* values. The samples in the PCA bi-plot followed a curvature, which was likely a result of the known non linear relation between the CIELab color values (Ayala et al. 1997, Pérez-Magarino and Gonzalez-Sanjose 2003). The similar positions in the bi-plot of the color parameters b*, Boulton's color values, H* and color intensity could also reflect, that only very young wines were included in the study and hence not included the aging effect on the color values. Although no distinct location of cultivars in the PCA was observed, some major differences could be observed between the cultivars. For instance Grenache and Cinsault produced lighter wines (high L*) with less color purity (low C*), while Alicante and Syrah produced darker wines (low L*) with more purple character (high b* and H*).

3.3.3 Prediction of wine color from grape phenolic profiles

Prediction of the wine color from the phenolic composition of the grapes requires that an accurate relation exist. Correlation coefficients were calculated to evaluate the linear relation between the individual phenolic levels in the grapes and the wine color values of the corresponding wines (Table 13).

Table 13. Correlation coefficients (r) between phenolic levels in the grapes and color parameters of the 54 wines (Jensen et al. 2008a).

Phenolic compound	WC total	WC copig.	WC PP	WC anth	L*	a*	b*	C*	H*	Tonality	Color intensity
Total phenols	0.85	0.83	**0.89**	0.84	**-0.86**	0.52	0.88	**0.70**	0.89	-0.63	0.86
Anth-spec	**0.95**	0.95	0.85	0.94	-0.85	0.31	0.89	0.56	0.90	-0.71	**0.95**
MP	0.91	0.92	0.79	0.91	-0.79	0.23	0.84	0.48	0.86	-0.66	0.91
SPP	0.82	0.82	0.77	0.81	-0.69	0.15	0.78	0.40	0.80	-0.47	0.82
LPP	0.60	0.59	0.64	0.59	-0.62	0.38	0.62	0.50	0.62	-0.48	0.61
Tannins	0.35	0.31	0.53	0.34	-0.51	**0.53**	0.40	0.54	0.40	-0.28	0.37
PP	0.80	0.79	0.80	0.78	-0.76	0.34	0.80	0.54	0.80	-0.56	0.80
Gallic acid	0.10	0.04	0.31	0.10	-0.20	0.32	0.21	0.31	0.20	0.00	0.11
(+)-catechin	-0.02	-0.07	0.18	-0.02	-0.12	0.37	0.11	0.32	0.10	0.07	0.00
(-)-epicatechin	0.13	0.08	0.32	0.14	-0.21	0.32	0.24	0.33	0.24	-0.01	0.14
Hydroxycin.	0.24	0.27	0.12	0.24	-0.07	-0.24	0.33	-0.07	0.34	0.03	0.24
Flavonols	0.77	0.78	0.73	0.76	-0.79	0.46	0.74	0.60	0.74	-0.62	0.77
Anth-HPLC	**0.95**	**0.96**	0.85	**0.95**	**-0.86**	0.34	**0.90**	0.59	**0.91**	**-0.74**	**0.95**

Bold values indicate the highest correlation coefficients for each wine color parameter.

Good correlations between several phenolic classes in the grapes and especially Boulton's color values, and color intensity were found, with anthocyanins and total phenols giving the best overall correlations (Table 13). This was in good accordance with the reported results using extensive extraction of grape phenols (Gonzalez-Neves et al. 2004, Iland 1987). For a*, C*, and tonality poorer direct relationships with the levels of grape phenols were observed (Table 13). The relationship between grape anthocyanins and Bolton's color values and color tonality was furthermore linear, as shown for total wine color (Figure 22).

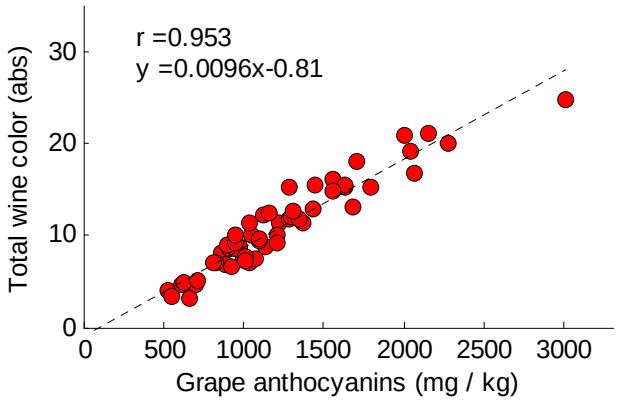


Figure 22. Direct relationship between grape anthocyanins and total wine color for the 54 wines (Jensen, unpublished data).

A further inspection of the direct relations showed, that at least lightness (L*), degree of redness (a*), and chroma (C* - data not shown) were not linear related to the anthocyanins in the grape (Figure 23). The non linear nature of the CIELab

parameters has been reported by others (Pérez-Magarino and Gonzalez-Sanjose 2003) and is also reflected in the way the CIELab values are calculated, involving both the use of transmittance values and cubic root transformations (Perez-Caballero et al. 2003).

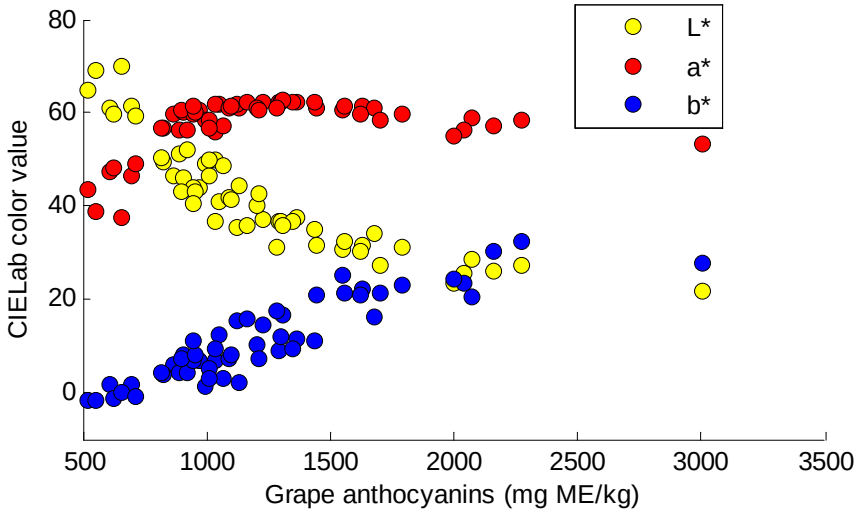


Figure 23. Direct relations between grape anthocyanins and CIELab color values of the corresponding wines (Jensen, unpublished data).

In order to be able to relate the phenolic content in the grapes with the CIELab color values of the corresponding wines a two step approach was attempted. First, selected absorbance values (450, 520, 570 and 630 nm) of the wines were predicted from the phenolic profiles of the grapes, since absorbance values were expected to be linear. Secondly, the predicted absorbance values of the wines were used to calculate the CIELab values, according to the described method (Perez-Caballero et al. 2003).

Partial least square regression was used to model the relation between the detailed phenolic profiles of the grapes and the wine color attributes, including the four selected absorbance values used to estimate the CIELab color values of the wines. The models were developed using a calibration set, and the performance of the models were evaluated both by segmented cross validation and test set validation. The performance of the models to predict the parameters was evaluated from the correlation coefficients, the RMSECV and RMSEP values, and the residual predictive deviation (RPD). The RPD value describes the proportion between the total variation of the samples and the standard error of prediction. A high RPD value indicates a good predictive ability of the model, and it has been reported that RPD values higher than three are considered to be good for predictive purposes (Cozzolino 2004). A more conservative, but also more detailed guideline of the predictive performances and applications according to the RPD values (Table 14) has been suggested (Williams 2001). The residual predictive deviation of the prediction set was in general higher than for cross validation, due to a slightly higher sample variation in the validation set (data not shown). Therefore evaluation of the predictive abilities will both be evaluated with the RPD values for both cross validation and test set validation.

Table 14. Classification of predictive performance and applications according to RPD values. Adapted from (Williams 2001).

RPD values	Predictive performance	Application
0.0-2.3	Very poor	Not recommended
2.4-3.0	Poor	Very rough screening
3.1-4.9	Fair	Screening
5.0-6.4	Good	Quality control
6.5-8.0	Very good	Process control
8.1 +	Excellent	Any application

Due to differences in units and consequently the scales of the concentration values for the different phenolic classes, auto scaling would ensure that all data was weighted the same in the model. However the best models were in general obtained when both X and Y data were mean centered (data not shown), and it was thus concluded, that an unequal weighting of the variables was advantageous for these models.

All four absorbance values (A450, A520, A570, and A630) of the wines were predicted quite well for both cross validation and the test set, with correlation coefficients 0.94 and 0.96 and RPD values between 2.9 and 3.4 (Table 15). These very similar correlation coefficients were likely a result of little variation in the shape of the wine spectra, due to the wines being produced under similar winemaking conditions and analyzed at the same time.

Table 15. Results for calibration and validation of estimation of wine color attributes from phenolic profiles of grapes using PLS regression (Jensen, unpublished data).

Wine color attribute	Calibration (N = 40)						Validation (N = 14)		
	Mean	SD	LV	r_{cv}	RMSECV	RPD_{cv}	r_{pred}	RMSEP	RPD_{pred}
A450	0.56	0.23	8	0.95	0.071	3.2	0.96	0.074	3.4
A520	1.08	0.47	8	0.95	0.15	3.1	0.95	0.16	3.2
A570	0.68	0.29	8	0.94	0.10	2.9	0.95	0.11	3.1
A630	0.12	0.05	8	0.95	0.017	3.1	0.95	0.018	3.3
WC total	11	4.8	8	0.95	1.57	3.0	0.96	1.46	3.6
WC copig	3.8	2.1	4	0.94	0.71	2.9	0.98	0.42	5.5
WC PP	1.71	0.57	1	0.89	0.26	2.2	0.92	0.27	2.4
WC anth.	5.5	2.2	8	0.94	0.77	2.8	0.96	0.66	3.5
L*	41	11	1	0.88	5.0	2.1	0.90	6.1	2.3
a*	58	5.1	2	0.42	4.6	1.1	0.59	6.2	1.2
b*	11	8.5	1	0.90	3.6	2.3	0.84	5.6	1.8
C*	60	5.7	1	0.62	4.4	1.3	0.74	6.0	1.4
H*	10.4	7.9	1	0.91	3.3	2.4	0.87	4.7	1.9
Tonality	0.47	0.04	1	0.58	0.03	1.2	0.79	0.03	1.6
Color intensity	1.58	0.65	8	0.95	0.21	3.1	0.96	0.22	3.4

PLS regression was carried out on mean centered data. Calibration was performed on 40 samples with cross validation in 10 continuous segments. Validation was performed on 14 external samples, selected to include all cultivars (1 random sample of each cultivar, except for Merlot with 7 random samples). One outlier was removed (Alicante), which due to a very high total wine color gave inaccurate b*, H*, and C* values.

Color intensity (Sudraud 1958) and Boulton's wine color values (Levengood and Boulton 2004), except wine color due to polymeric pigments were also predicted well (Table 15). As a consequence of the non linear nature (Figure 23) of especially the two CIELab color values: a* and C*, these were not predicted very well by the PLS model. The poor prediction of tonality (RPD = 1.2 – 1.6, Table 15) could be a

consequence of the very small variation of this parameter (between 0.41 and 0.55, cf. Table 12).

Instead of predicting the CIELab color values directly, the predicted absorbance values (A450, A520, A570, and A630) were used to calculate the CIELab values using the reported method (Perez-Caballero et al. 2003). By this approach it was possible to allow prediction of L*, b* and C* (RPD between 3.2 and 4.2), and to some extent also a* and C* color values (RPD between 2.3 and 3.7) (Table 16). It was hence concluded, that using an indirect approach for predicting the CIELab color values allowed compensation of non linear nature and gave better models. However, the indirect approach for calculation of the CIELab color values require that the absorbance values are accurately predicted from the phenolic composition of the grapes, since errors will be greatly magnified in the CIELab calculations.

Table 16. Results for CIELab values calculated from the absorbance values (A450, A520, A570, and A630) predicted from the detailed phenolic profiles of grapes (data was mean centered), (Jensen, unpublished data).

Color attribute	Calibration set (N = 40)					Validation set (N = 14)		
	Mean	SD	r_{cv}	RMSECV	RPD	r_{pred}	RMSEP	RPD
L*	41	10.7	0.95	3.3	3.2	0.97	3.3	4.2
a*	58	5.1	0.93	2.2	2.3	0.95	2.3	3.3
b*	11	8.5	0.96	2.5	3.4	0.95	3.0	3.4
C*	60	5.7	0.93	2.3	2.4	0.96	2.3	3.7
H*	10	7.9	0.96	2.3	3.5	0.95	2.8	3.3

The best correlation coefficients found for the direct relationships between the concentrations of the individual phenolic compounds (mainly anthocyanins by HPLC) and several of the wine color attributes (Table 13) were of similar magnitude as for the PLS regression, using the detailed phenolic profile (Table 15). Hence, it was suspected that the concentration of anthocyanins provided the most relevant information to allow prediction of at least some of the wine color attributes. Only using the grape anthocyanins instead of the detailed phenolic profiles, to indirectly predict the CIELab color values gave however slightly poorer predictions (Table 17) than for the detailed profiles (Table 16).

On the other hand, only the levels of anthocyanins seemed to be sufficient to obtain a prediction of color intensity, Boulton's wine color parameters (except wine color due to polymeric pigments), and the CIELab parameters: L*, b*, and C* (Table 17). The predictive residual deviation (RPD) values for these color attributes were between 2.4 and 5.7, which shows some predictive ability that can mainly be used for screening purposes (Williams 2001).

Table 17. Estimated CIELab values calculated from the predicted absorbance values (A450, A520, A570, and A630) and predicted Boulton's and Sudraud's color values from grape anthocyanin levels, measured by HPLC (all data is mean centered). (Jensen, unpublished data).

	Calibration set (N = 40)					Validation set (N = 14)		
Color attribute	Mean	SD	r_{cv}	RMSECV	RPD	r_{pred}	RMSEP	RPD
A450	0.56	0.23	0.93	0.084	2.7	0.97	0.064	4.2
A520	1.08	0.47	0.93	0.178	2.6	0.98	0.108	5.1
A570	0.68	0.29	0.90	0.129	2.3	0.97	0.084	4.0
A630	0.12	0.05	0.92	0.022	2.5	0.96	0.016	3.7
L*	41	10.7	0.92	4.3	2.5	0.97	4.0	3.8
a*	58	5.1	0.81	3.2	1.7	0.95	3.2	2.4
b*	11	8.5	0.91	3.6	2.4	0.93	3.6	2.7
C*	60	5.7	0.81	3.5	1.7	0.95	3.4	2.6
H*	10	7.9	0.92	3.1	2.6	0.94	3.1	2.9
WC total	10.9	4.8	0.93	1.78	2.7	0.98	1.2	4.8
WC copig.	3.79	2.09	0.94	0.73	2.8	0.98	0.40	5.7
WC PP	1.71	0.57	0.80	0.34	1.6	0.89	0.32	2.2
WC anth.	5.45	2.18	0.92	0.84	2.6	0.98	0.55	4.7
Tonality	0.47	0.04	0.60	0.03	1.2	0.91	0.02	2.2
Color intensity	1.58	0.65	0.93	0.25	2.6	0.98	0.16	4.8

3.3.4 Discussion, conclusion, and future perspectives

This study showed, that several color attributes of freshly fermented pH normalized red wines produced under experimental conditions could be predicted from the phenolic profiles of the corresponding grapes.

The levels of some individual phenolic compounds, especially anthocyanins and total phenols, in the grapes were found to correlate well with several wine color attributes. The direct correlation between grape anthocyanins and the color intensity of the wines ($r^2 = 0.91$) found in this study was similar to previous results ($r^2 = 0.82$) using an extensive grape extraction protocol (Iland 1987). A good correlation between grape anthocyanins and wine color intensity have also been reported using Glories' extractability method ($r^2 = 0.95$), but it did not seem to be necessary to use the information on anthocyanin extractability (Gonzalez-Neves et al. 2004).

The prediction of several other wine color attributes from the phenolic composition of grapes was investigated to provide further information of the wine color than color intensity. Due to the known impacts of other phenols on the wine color, in particular due to copigmentation and formation of polymeric pigments, a multivariate approach using a detailed phenolic profile of the grapes was attempted for the prediction of the wine color attributes. Although some wine color attributes were predicted well using a multivariate approach, it was in most instances sufficient to just use the anthocyanin levels to predict the wine color attributes. Grape anthocyanins levels allowed acceptable predictions of the following wine color attributes: Color intensity, Boulton's color values (except color due to polymeric pigments), and some CIELab color values (L*, b*, and C*).

Due to the non linear nature of the CIELab wine color values these were best predicted from the phenolic composition of the grapes when an indirect approach was used, by first predicting absorbance values of the wines and using this information to calculate the CIELab color values. However, this approach was also found to be sensitive to small errors, due to the complex formulas for calculating the CIELab

values. It has been proposed that the description of wine color using the CIELab system could be more appropriate with regards to how color is perceived (Ayala et al. 1999). However, it has also been demonstrated that CIELab color values from transmission and reflectance measurements are very different (Martinez et al. 2001), and it still needs to be verified how accurate CIELab color values relate with perceived wine color.

It is well known that the winemaking conditions have an important impact on the phenolic extraction from the grapes (Sacchi et al. 2005). Since the prediction of wine color attributes were based on standardized winemaking conditions, changing the conditions will of course make the predictions less accurate. However, knowing what to expect under certain condition would allow the winemaker to make decisions about the best conditions for the individual grape loads. For instance, if the color of the wine was predicted to be very low it could be useful to use an extended maceration time .

The extraction method in this study shows that it will be possible to use a fast extraction method for evaluation of the phenolic composition of red grapes and use this information for predicting the color attributes of the corresponding wine.

Future perspectives
This work has been carried out under experimental conditions including: Micro scale fermentations, frozen grape material, and not considering aging of the wines. Future work should focus on verifying whether the results found in this study will also apply to commercial wine making conditions.

3.4 Quantification of polyphenols and wine color by FT-MIR spectroscopy (Paper II and Paper IV)

3.4.1 Introduction and scope

In the two previous sections it was found, that the phenolic composition of red grapes allowed prediction of at least some color attributes and phenolic levels in the corresponding wines. However, quantification of the phenolic composition typically requires different analytical methods, extensive sample handling and long analysis times. The objective of this work was to study the feasibility of using FT-MIR spectroscopy for quantification of the phenolic composition of grape extracts and wines.

The use of FT-MIR spectroscopy has found great utility for the routine analysis of various foods including grape juices and wines (Andersen et al. 2002). One major advantage of FT-MIR analysis is that reliable results are obtained very fast (typically within one minute) and that several components are determined simultaneously.

The application of FT-MIR spectroscopy for quantification of grape and wine phenols presents some major challenges. Phenols are only present in low concentrations in grapes and wines, and thus only contribute little to the signals in the infrared spectra. Overlapping signals in the infrared spectra from other organic compounds will further interfere with signals from the phenols and influence how well the phenols can be measured. Overlapping signals of the different phenolic classes will most likely also affect the calibration models.

Realizing these challenges, the objective of this study was to investigate how well different classes of phenols in grapes and wines could be measured using FT-MIR spectroscopy. Since tannins are the most abundant group of phenols found in wines (Kennedy et al. 2006b), but yet are difficult to measure directly without various pretreatment reactions of the sample, see below, it was first attempted to quantify the concentration of tannins in commercial wines directly by FT-MIR. The investigation also involved the study of a reference method for quantification of tannins by protein precipitation. Secondly the analysis of other less abundant polyphenols in wines and grapes were investigated and it was attempted to address how the analytical results could be used for prediction of color attributes or phenolic composition of wine.

3.4.2 Analysis of red wine tannins by protein precipitation (Paper II)

Even though tannins are typically the most abundant class of polyphenols in red wines, quantification is difficult due to the diversity of this class of polyphenols. Tannins have the ability to precipitate with proteins in saliva, which is believed to be the main reason for the astringent sensation of red wines (Gawel 1998). An analytical method for tannin quantification by protein precipitation with bovine serum albumin was recently reintroduced (Harbertson et al. 2003) and has been recommended for applications within winery settings (Harbertson and Spayd 2006). The method relies on that tannins can be precipitated with bovine serum albumin, which is then separated by centrifugation, redissolved, and finally measured from a color reaction with ferric chloride (Hagerman and Butler 1978, Harbertson et al. 2003). Furthermore wine tannin concentrations quantified by protein precipitation with bovine serum albumin have been found to correlate particular well with wine astringency (r^2= 0.82), when compared with other analytical methods (Kennedy et al. 2006a).

Although the mechanism for the precipitation between tannins and proteins is not fully understood, it has been reported that precipitation is influenced by threshold levels before precipitation occurs (Hagerman and Butler 1978, Hagerman and Robbins 1987). This could have an impact on the reliability of the results of this assay, especially when samples are diluted differently. The objective of this work was to investigate the effect of sample dilution on the reliability of tannin analysis by protein precipitation (Jensen et al. 2008c).

The tannin response was measured as the absorbance after color reaction with ferric chloride subtracting the background absorbance. The linearity of the tannin response was evaluated by analyzing five commercial wines at different dilutions (from undiluted to 10 times dilution) and plotting the tannin response against the inverse dilution factor (Figure 24). Linear relationships ($r > 0.999$) could be established for all wines in parts of the dilution ranges. However, for some of the wines giving high tannin responses the linear relationship was not valid at low dilutions, likely caused by insufficient protein for the precipitation. On the other hand, all regression lines were found to give negative y-intercepts (see legends in Figure 24), indicating the existence of a threshold level of tannin before precipitation would occur.

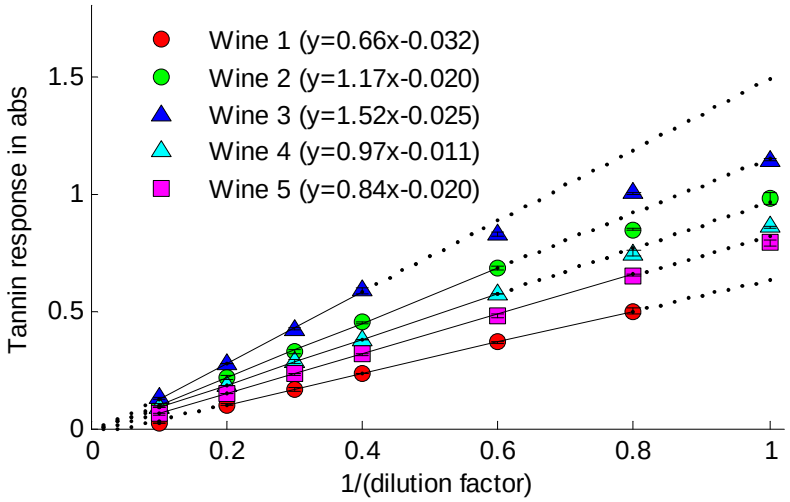


Figure 24. Relation between the inverse dilution factor and the tannin response (± SD) of five commercial wines (Jensen et al. 2008c). Regression lines were calculated for the observed linear range (solid lines, see legend for functions) and expanded to the non linear range (dotted lines).

It was found that both the precipitation threshold and the non linear nature of the tannin response at higher tannin concentrations caused underestimation of the tannin concentration. The maximum determined tannin concentration for each wine was used to benchmark the tannin concentrations determined at the different dilutions as a percentage of the maximum. This allowed us to evaluate how much the tannin concentrations were underestimated as a function of the tannin response (Figure 25).

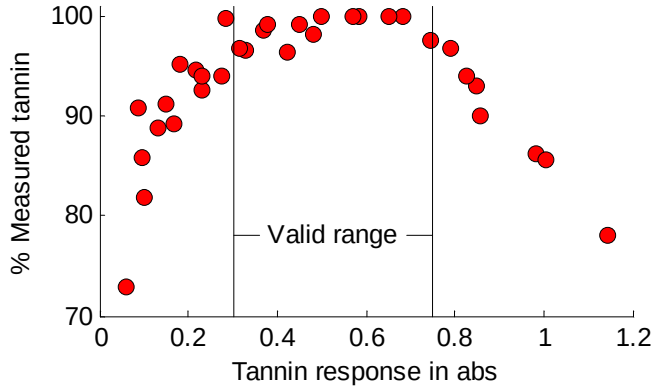


Figure 25. Valid range (95 – 100 %) of the tannin response defined from the relative proportion of measured tannin concentration to the maximum determined tannin concentration (Jensen et al. 2008c).

By allowing a 5 % underestimation of the tannin concentration it was found that the tannin response should lie between 0.3 and 0.75 absorbance units.

3.4.3 Identification of spectral regions for quantification of wine tannins by FT-MIR spectroscopy (Paper IV)

Since tannins are the most abundant class of polyphenols found in red wine, it was decided to investigate how well tannins could be measured by FT-MIR spectroscopy. The purpose of the examination was also to obtain an assessment of whether FT-MIR spectroscopy would be suitable for the measurement of polyphenols in red wine. Measurement of tannins in grapes and wines has received much attention, due to the impact of tannins on the mouth-feel properties and color stability of red wines (Gawel

1998, Kennedy et al. 2006a, Singleton and Trousdale 1992). The development of a rapid analytical technique for tannin measurement would therefore be a valuable tool for routine analysis at wineries.

Chemometrical techniques, such as partial least squares (PLS) regression, are typically used to develop multivariate calibration models from spectral data. However, as already mentioned above (section 3.4.1.), quantification of components in low concentration, such as tannins, is difficult due to spectral interferences from other wine components. Such overlapping spectral signals can lead to suboptimal PLS models, and selection of the relevant spectral regions may improve the performance of the calibration models (Norgaard et al. 2000).

A set of 128 commercial red wines were analyzed by FT-MIR spectroscopy and the tannin concentrations were measured. The wines were selected to represent considerable variation in the age (11 vintages), the grape varieties (at least 30 varieties), and production countries (16 countries). The concentration of tannins in the wines ranged from 92 to 1060 mg CE/L and thus covered the most typical concentration range of tannins in commercial red wines reported by others (Fernandez and Agosin 2007, Heredia et al. 2006, Skogerson et al. 2007, Versari et al. 2006). A list of the wines and their tannin levels is given in the supplementary material included for paper IV (enclosed with the thesis).

Spectral regions for tannin quantification

The non informative and noisy regions of the FT-MIR spectra were removed, leaving the 'good range' region (2969 to 2699 cm^{-1}, 1812 to 1716 cm^{-1}, and 1577 to 933 cm^{-1}). The fingerprint region was defined between 1577 and 933 cm^{-1} and the main region for phenolic absorptions was identified to be approximately between 1577 and 1157 cm^{-1}. In addition different variable selection methods were used to find the most relevant spectral regions in the good range region for tannin quantification. The methods included synergistic interval PLS (si-PLS), backward interval PLS (bi-PLS), genetic algorithm PLS (GA-PLS), and a newly developed selection method: iterative backward elimination changeable size interval PLS (IBECSI-PLS) (Jensen et al. 2008b). As opposed to many of the existing variable selection methods, the intervals to be eliminated in IBECSI-PLS were found by stepwise expansions of the regions to be removed. Variable selections were performed on the calibration set allowing up to 10 latent variables, and the regions giving the optimal calibration models were selected.

The spectral regions identified either manually or by the variable selections were compared with the characteristics infrared spectra of a red wine, oak tannin, (+)-catechin, and grape tannin (Figure 26). Although the regions identified by the four variable selection methods were not identical, two regions were selected by all four methods: One region from 1060 to 995 cm^{-1}, dominated by the high absorption of ethanol and one region between 1485 and 1425 cm^{-1}, with a characteristic signal from grape tannin (Figure 26), which corresponded to an aromatic ring stretch (Shurvell 2002). All variable selection methods also included wavelengths between 2969 and 2699 cm^{-1}, which could not be ascribed to any spectral features of tannins, and hence might function as a reference point in the spectra. A distinct peak at 1285 cm^{-1} has been reported to correspond to the C-O stretch of flavonoid pyran ring structure (Edelmann and Lendl 2002), but was in neither case selected by any of the four

methods. Both bi-PLS and IBECSI-PLS retained wavelengths around 1750 cm^{-1}, where oak tannin had a signal, which was likely ascribed to the C=O group found in hydrolyzable tannins.

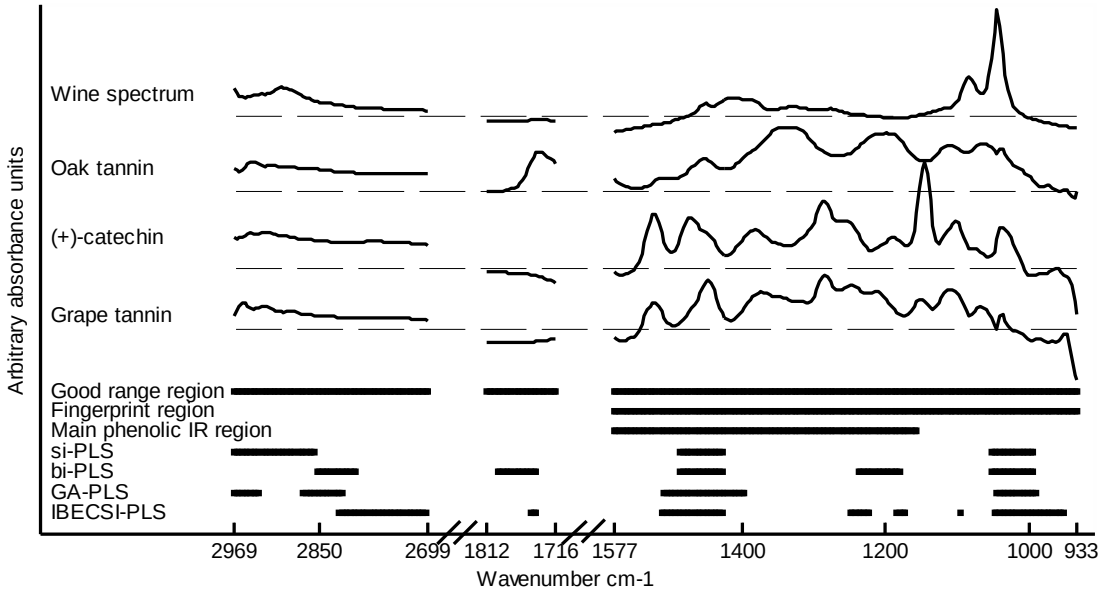


Figure 26. Identified spectral regions for tannin quantification obtained by different variable selection procedures in relation to the IR spectrum of a wine sample (scaled down 100 times) and the IR signals of oak tannin, (+)-catechin and commercial grape tannin (Jensen et al. 2008b).

Quantification of red wine tannins

Calibration models were developed for each of the seven spectral regions identified in Figure 26 using up to 10 latent variables. The performances of the models using the different regions were evaluated from the predictive ability on the independent test set (Table 18). It was concluded, that the better models were obtained using the manually selected intervals, than the whole good range region. The spectral regions identified by variable selections further improved the performance of the calibration models with RMSEP values between 69 and 79 mg CE/L. Only small differences in the RMSEP values were observed for the models derived from the four spectral regions identified by variable selection.

Table 18. Calibration and validation results for tannin quantification in red wines by FT-MIR spectroscopy from different spectral regions (Jensen et al. 2008b).

Spectral region	# Var [a]	LV [b]	RMSEC [c]	RMSECV [c]	RMSEP [c]	r_{val} [d]
good range region	265	10	65	92	115	0.87
fingerprint region	168	10	69	91	92	0.91
main phenolic region	110	10	54	75	88	0.90
si-PLS region	62	10	53	65	77	0.93
bi-PLS region	78	10	53	65	69	0.94
GA-PLS region	70	9	55	69	79	0.93
IBECSI-PLS region	97	10	49	59	75	0.94

[a] Number of variables. [b] Number of latent variables. [c] Root mean square error of calibration, cross validation, and prediction respectively in mg CE/L. [d] Correlation coefficient between the measured and the predicted tannin levels.

An acceptable correlation between the predicted and measured concentration of tannins using the spectral region identified by IBECSI-PLS was found (Figure 27). The prediction performance of the developed model (RMSEP = 75 mg CE/L; r =

0.94) was similar to reported values (RMSECV = 63; r = 0.99) also using FT-MIR spectroscopy but only 20 samples and a large number of latent variables and no independent validation of the model (Versari et al. 2006). Quantification of tannins in red wines by FT-MIR using attenuated total reflection (ATR) has been reported to give better results (RMSEP = 51 mg/L; r = 0.96), but requires extensive sample preparation by solid phase extraction and solvent evaporation (Fernandez and Agosin 2007). UV/VIS spectroscopy has recently been reported suitable for quantification of tannins (RMSEP = 66 mg CE/L; r = 0.93) and also other phenolic classes, including polymeric pigments and anthocyanins in fermenting musts and finished red wines (Skogerson et al. 2007).

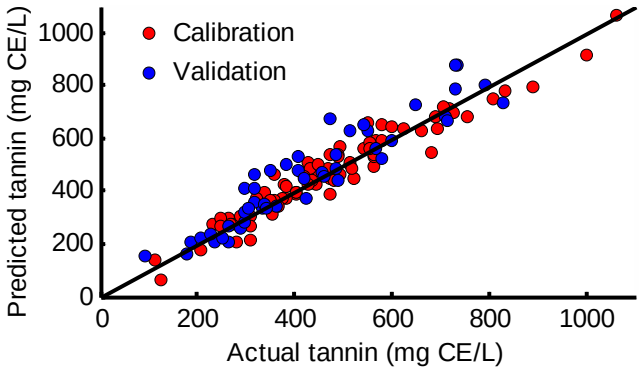


Figure 27. Correlation between the measured tannin concentration and the predicted tannin concentration from the spectral region identified by IBECSI-PLS (Jensen et al. 2008b).

Measurement of other wine phenolics by FT-MIR spectroscopy

Since tannins are the most abundant class of phenolics in wines, these were assumed to be the easiest class to quantify by FT-MIR spectroscopy. Quantification of less abundant classes of phenols was therefore expected to be more challenging. A more detailed analysis of the 128 wines was carried out to evaluate the feasibility of FT-MIR spectroscopy for quantification of other phenolic classes in commercial red wines. Backward interval PLS was used to optimize the calibration models using the 81 wines. The performances of the calibration models were evaluated with an independent set of wines. By comparison of the correlation coefficients and residual predictive deviation values it was concluded that tannins were measured better than any of the other phenolic classes under investigation (Table 19).

Table 19. Calibration and validation results for measurement of phenols in commercial wines from FT-MIR by PLS regression (cross validation in 10 segments and up to 10 latent variables). All data was mean centered and backwards interval PLS was used to select important variables (Jensen, unpublished data).

Phenolic class	Number of variables	Calibration (N=81)						Validation (N=47)		
		LV	Mean	SD	RMSECV	r_{cv}	RPD	RMSEP	r_{pred}	RPD
MP (abs)	94	10	1.07	0.49	0.31	0.78	1.6	0.33	0.78	1.4
PP (abs)	93	10	2.18	0.84	0.45	0.85	1.9	0.50	0.86	1.6
LPP (abs)	78	10	1.07	0.49	0.33	0.75	1.5	0.32	0.76	1.4
SPP (abs)	79	10	1.29	0.49	0.20	0.91	2.5	0.23	0.92	2.2
Tannins (mg CE/L)	78	10	472	180	65	0.93	2.8	69	0.94	2.7
Anthocyanins (mg ME/L)	46	10	73	58	37	0.77	1.5	52	0.62	0.9

However the RPD values for tannins of about 2.8 would still only classify as a relatively poor prediction and would be recommended to be used for rough screening purposes according to the guidelines – see section 3.3.3. (Table 14). Nevertheless the FT-MIR tannin measurements does provide some useful information, which otherwise may not be available.

From the validated residual prediction deviation values it was concluded that quantification of the remaining phenolic classes, including anthocyanins, monomeric pigments, polymeric pigments, and large polymeric pigments were not possible. This was in contrast with recently published results where anthocyanins concentrations of young wines were quantified with an acceptable validated correlation coefficient ($r_{pred}^2 = 0.92$) using a Winescan FT120 FT-MIR instrument (Soriano et al. 2007). These conflicting results could likely be a result of the large variation in the ages of the wines included in this study, while Soriano et al. used very young wines from the same vintage. It is well known that the anthocyanin content of wines rapidly decreases as it ages, due to chemical transformation of anthocyanins (Ribéreau-Gayon et al. 2006). It is thus likely that the inability of FT-MIR spectroscopy to quantify anthocyanins was due to a combination of the low concentrations of anthocyanins found in aged wines and interferences from the anthocyanin derivatives formed during aging (such as polymeric pigments). The recent applications of UV/VIS spectroscopy on fermenting musts and young wines for quantification of tannins and pigments seemed more appropriate than FT-MIR spectroscopy regarding quantification of the different types of pigments (Skogerson et al. 2007). The UV/VIS models did however not include much variation in the ages of the wines, and a larger variation in the age of the wines would likely alter the results due to the complex pigment changes occurring during maturation.

3.4.4 Measurement of polyphenols in grapes and young wines by FT-MIR spectroscopy

Even though it was found that the phenolic composition of commercial red wines was difficult to quantify with FT-MIR spectroscopy, the concentration of phenolics were higher in the grape extracts, than in the commercial wines. At the same time the phenolic composition of grapes are less complex than aged wines, which might allow better quantification from FT-MIR spectra for grapes.

Quantification of both total phenols and tannins in the grape extracts by FT-MIR spectroscopy were possible, with RPD values higher than 3 (Table 20).

Table 20. Calibration and validation results for selected grape phenols from FT-MIR spectra of grape extracts by PLS regression (mean centering, cross validation in 10 segments, and up to 10 latent variables). FT-MIR spectra in the main phenolic region (110 variables from 1577 to 1157 cm^{-1}) were used for regression (Jensen, unpublished data).

Phenolic class (grape extracts)	Calibration (N=40)					Validation (N=14)		
	LV	Mean	RMSECV	r_{cv}	RPD	RMSEP	r_{pred}	RPD
Total phenols (0.01 abs)	10	1508	66	0.98	5.0	106	0.96	3.6
Tannins (mg CE/kg)	10	2711	204	0.95	3.2	218	0.97	4.1
PP (abs)	7	0.96	0.22	0.75	1.5	0.31	0.72	1.4
Anth-HPLC (mg ME/kg)	10	1219	177	0.92	2.6	263	0.88	2.1

With RPD values 2.1 and 2.6 anthocyanins were not quantified well. Quantification of the levels of polymeric pigments in grape extracts was not possible, probably due to the low occurrence of this phenolic class in grapes.

Quantification of the main phenols in young red wines by FT-MIR was slightly better than for grape extracts – as evaluated from the value of the RPD values (Table 21). Tannins and total phenols were thus in fact quantified very well in the red wines with RPD values higher than 5. Anthocyanins and polymeric pigments could be quantified to some extent with RPD values between 2.3 and 2.9. For both grapes and wines, it did not seem possible to quantify the less abundant classes of phenols by FT-MIR spectroscopy (data not shown).

Table 21. Calibration and validation results for selected phenols in young wines from FT-MIR spectra of the wines by PLS regression (mean centering, cross validation in 10 segments, and up to 10 latent variables). FT-MIR spectra in the main phenolic region (110 variables from 1577 to 1157 cm^{-1}) were used for regression (Jensen, unpublished data).

Phenolic class (wines)	Calibration (N=40)					Validation (N=14)		
	LV	Mean	RMSECV	r_{cv}	RPD	RMSEP	r_{pred}	RPD
Total phenols (0.01 abs)	7	666	32	0.98	5.3	31	0.99	5.8
Tannins (mg CE/kg)	10	886	61	0.98	5.3	58	0.98	5.3
PP (abs)	9	0.95	0.14	0.90	2.3	0.13	0.92	2.6
Anth-HPLC (mg ME/kg)	6	380	50	0.92	2.5	52	0.94	2.9

Since at least some wine phenolics could be predicted from the phenolic composition of the grape phenols (Section 3.2), it might also be possible to predict the phenolic composition of the young wines from the infrared spectra of the grapes. Using PLS regression it was to some extent possible to predict the levels of total phenols and polymeric pigments of the wines from the infrared spectra of grape extracts (Table 22). The prediction of polymeric pigments was better than expected, and might reflect that the formation of these pigments was related to the chemical composition of the grapes. On the contrary it was not possible to predict anthocyanins or tannins levels of wines from the FT-MIR spectra of grapes, which was likely a consequence of the poor direct relation between grape and wine tannins (Table 9) and that grape anthocyanins could not be measured very accurately (Table 20). Thus, prediction of the phenolic composition of wine phenols from FT-MIR spectra of grape extracts requires both a representative extraction of the grapes and the ability of FT-MIR to measure the phenolic compounds.

Table 22. Calibration and validation results for selected phenols in young wines from FT-MIR spectra of the corresponding grape extracts by PLS regression (mean centering, cross validation in 10 segments, and up to 10 latent variables). FT-MIR spectra in the main phenolic region (110 variables from 1577 to 1157 cm^{-1}) were used for regression (Jensen, unpublished data).

Phenolic class (wines)	Calibration (N=40)					Validation (N=14)		
	LV	Mean	RMSECV	r_{cv}	RPD	RMSEP	r_{pred}	RPD
Total phenols (0.01 abs)	10	666	59	0.94	2.9	78	0.91	2.4
Tannins (mg CE/kg)	6	886	173	0.85	1.9	200	0.80	1.5
PP (abs)	9	0.95	0.14	0.90	2.3	0.11	0.95	3.1
Anth-HPLC (mg ME/kg)	10	380	51	0.91	2.4	90	0.80	1.6

3.4.5 Prediction of wine color attributes from FT-MIR spectra of grape extracts and wines

Prediction of wine color attributes directly from FT-MIR spectra of grapes or wines, would allow a fast evaluation of the color potential of the grapes for wine making and a fast measure of the realized wine color attributes, respectively. Wine color attributes were measured for the 54 freshly fermented wines after pH adjustment (pH 3.6). The predicted CIELab color values were calculated indirectly from the FT-MIR predicted absorbance values of the wines (A450, A520, A570, and A630) from either grape extracts or wines. Boulton's and Sudraud's color attributes (Levengood and Boulton 2004, Sudraud 1958) were predicted directly from the FT-MIR spectra of either grape extracts or wines.

Prediction of wine color attributes from FT-MIR spectra of the grape extracts were to some extent possible (Table 23). The best prediction results were obtained for Boulton's color values, color intensity, lightness (L*), degree of blueness (b*), and hue angle (H*). The prediction of these wine color attributes from FT-MIR of grapes was not as good as using the detailed phenolic profiles of grapes (cf. Table 15 and Table 16). With RPD values between 2.1 and 4.3, the prediction performances would be suited for screening purposes, according to the RPD value guidelines (Table 14).

Table 23. Calibration and validation results for prediction of wine color attributes from FT-MIR spectra of grape extracts by PLS regression (mean centering, cross validation in 10 segments, and up to 10 latent variables). FT-MIR spectra in the main phenolic region (110 variables from 1577 to 1157 cm^{-1}) were used for regression (Jensen, unpublished data).

Color attribute	Calibration set (N = 40)					Validation set (N = 14)		
	LV	Mean	r_{cv}	RMSECV	RPD	r_{pred}	RMSEP	RPD
A450	10	0.56	0.95	0.07	3.3	0.93	0.09	2.8
A520	10	1.08	0.94	0.15	3.0	0.91	0.23	2.4
A570	10	0.68	0.94	0.10	2.9	0.91	0.14	2.4
A630	10	0.12	0.93	0.02	2.8	0.92	0.02	2.5
L*	- [a]	41	0.91	4.33	2.4	0.91	5.63	2.5
a*	- [a]	58	0.79	3.29	1.5	0.78	5.01	1.5
b*	- [a]	11	0.94	2.82	3.0	0.92	4.09	2.5
C*	- [a]	60	0.82	3.39	1.7	0.81	5.12	1.6
H*	- [a]	10	0.94	2.70	2.9	0.92	3.68	2.6
WC total	10	11	0.94	1.6	3.0	0.91	2.2	2.5
WC copig.	10	3.8	0.93	0.75	2.7	0.88	1.1	2.1
WC PP	10	1.7	0.93	0.21	2.7	0.97	0.15	4.3
WC anth.	10	5.5	0.94	0.72	3.0	0.91	1.00	2.4
Tonality	4	0.47	0.59	0.03	1.2	0.53	0.04	1.0
Color intensity	10	1.6	0.95	0.21	3.1	0.92	0.30	2.5

[a] Values were calculated indirectly from the predicted absorbance values (A450, A520, A570, and A630) of the young wines from FT-MIR spectra of grape extracts.

Several wine color attributes were predicted well from the FT-MIR spectra of the wines, in particular Boulton's color values and color intensity (Table 24). It was possible to predict the he CIELab color values: L*, b*, and H* directly from the FT-MIR spectra (Table 24). RPD values for the mentioned color attributes were between 2.5 and 7.3, but since the cross validated RPD values were not higher than 3.7 it was concluded that the predictions were mainly suitable for screening purposes.

Table 24. Calibration and validation results for prediction of wine color attributes from FT-MIR spectra of the wines by PLS regression (mean centering, cross validation in 10 segments, and up to 10 latent variables). FT-MIR spectra in the main phenolic region (110 variables from 1577 to 1157 cm^{-1}) were used for regression. CIELab color values were predicted directly from the FT-MIR spectra (Jensen, unpublished data).

Color attribute	Calibration set (N = 40)					Validation set (N = 14)		
	LV	Mean	r_{cv}	RMSECV	RPD	r_{pred}	RMSEP	RPD
A450	10	0.56	0.97	0.06	3.9	0.99	0.06	5.9
A520	10	1.08	0.96	0.13	3.6	0.99	0.12	6.3
A570	7	0.68	0.95	0.09	3.2	0.96	0.10	3.5
A630	7	0.12	0.93	0.02	2.8	0.96	0.02	3.5
L*	7	41	0.94	3.7	2.9	0.95	4.7	3.0
a*	3	58	0.42	4.6	1.1	0.80	5.3	1.4
b*	7	11	0.92	3.4	2.5	0.92	4.1	2.6
C*	4	60	0.64	4.4	1.3	0.80	5.5	1.5
H*	7	10	0.93	3.0	2.6	0.94	3.4	2.9
WC total	10	11	0.96	1.4	3.5	0.99	1.1	6.7
WC copig.	7	3.8	0.94	0.70	3.0	0.96	0.8	3.5
WC PP	9	1.7	0.96	0.17	3.4	0.95	0.19	3.3
WC anth.	9	5.5	0.96	0.59	3.7	0.99	0.35	7.3
Tonality	7	0.47	0.82	0.02	1.7	0.85	0.02	1.8
Color intensity	10	1.6	0.96	0.18	3.7	0.99	0.16	6.5

This time the CIELab values were not estimated well by first predicting the absorbance values (A450, A520, A570, and A630 - Table 24) from the FT-MIR spectra of the wines (Table 25). A close inspection of the results showed that a few wines with low color absorbance values, mainly in the calibration set, were predicted poorly (data not shown). This showed that although in some cases the indirect prediction of the CIELab values via the predicted absorbance values gave better predictions (Table 15 and Table 16) this method was also sensitive to prediction errors of some of the extreme samples. A solution to this might be to only use the indirect method for wines within certain absorbance values, but should be validated further before implementation.

Table 25. Calculated CIELab values from predicted absorbance values (A450, A520, A570, and A630 – see Table 24), (Jensen, unpublished data).

Color attribute	Calibration set (N = 40)				Validation set (N = 14)		
	Mean	r_{cv}	RMSECV	RPD	r_{pred}	RMSEP	RPD
L*	41	0.93	4.8	2.2	0.96	4.93	3.0
a*	58	0.92	3.3	1.5	0.98	3.07	2.5
b*	11	0.82	5.3	1.6	0.76	6.74	1.4
C*	60	0.91	2.8	2.0	0.98	2.30	3.8
H*	10	0.64	7.6	1.0	0.48	9.63	0.9

3.4.6 Discussion, conclusion, and future perspectives

This work demonstrated that it to some extent was possible to quantify some of the phenolic compounds in grape extracts and wines with FT-MIR spectroscopy. The best quantifications were obtained for total phenols and tannins in the grape extracts and young wines. This was in good accordance with these two phenolic classes being the most abundant phenolic classes in grapes and red wines. Quantification of both tannins and total phenols in the young wines with FT-MIR was very accurate and better than the reported results with UV/VIS spectroscopy of young red wines (Skogerson et al. 2007). Tannins could also be quantified in commercial red wines

with FT-MIR spectroscopy, but with a lower accuracy. The quantification of tannins in grape extracts and young wines with FT-MIR spectroscopy were considerably better than results reported for the prediction strength of VIS/NIR spectroscopy analyses on red wines (RPD = 1.8) (Cozzolino et al. 2004), but quite similar to UV/VIS spectroscopy (Skogerson et al. 2007).

Anthocyanins, being the phenolic class of primary interest for wine color, could to some extent be quantified in grape extracts and young wines by FT-MIR spectroscopy. However, it was not possible to quantify anthocyanins in commercial wines with FT-MIR spectroscopy. This finding was likely due to spectral interferences from other phenolics and the low concentrations of anthocyanins in aged wines. This result was in contrast with other reports on the use of FT-MIR for quantification of anthocyanins (Soriano et al. 2007) and was probably due to the great variation in the wine ages employed in the present study. Good results of quantification of anthocyanins in fermenting must and young red wines both by NIR/VIS (Cozzolino et al. 2004) and UV/VIS (Skogerson et al. 2007) have been reported. Both of these techniques look very promising, but their validities have not yet been verified with aged wines. Quantification of anthocyanins in grapes by NIR/VIS spectroscopy of grape extracts (Gishen et al. 2005, Janik et al. 2007) was also concluded to be more accurate than the prediction of anthocyanins in grape extracts by FT-MIR spectroscopy in the present study. The apparently better predictions of anthocyanins by UV/VIS or NIR/VIS spectroscopy may very well be related to the strong absorptions of anthocyanins in the visible regions.

Furthermore, prediction of phenolic compositions or color of the wines from the FT-MIR spectra of grapes was investigated. Total phenols and polymeric pigments in the young wines could to some extent be predicted from the FT-MIR spectra of the grape extracts, while tannins and anthocyanins were not predicted so well. On the other hand, several wine color attributes could be predicted from the FT-MIR spectra of both the grape extracts and the wines. The predictive performances of the models indicated that the results could mainly be used for screening purposes. Nevertheless the prediction of wine color attributes from FT-MIR analysis of grapes could be a valuable tool for evaluation of the wine color potential of the grapes.

Future perspectives

The recent advances within UV/VIS spectroscopy for quantification of phenolic compounds in fermenting must and young wines (Skogerson et al. 2007) suggest that this technology gives a better accuracy for quantification of the different pigments in grapes and wines. An interesting investigation would be to compare the ability of UV/VIS with FT-MIR for the same samples and investigate if UV/VIS and FT-MIR could be combined for a more versatile analytical capability. In this way the strengths of FT-MIR for the analysis of e.g. carbohydrates, ethanol, organic acids, and pH could be supplemented with the UV/VIS strengths for analysis of pigments and other phenols.

CHAPTER 4 CONCLUSIONS AND FUTURE PERSPECTIVES

Prediction of the phenolic compositions and color attributes of red wines from the phenolic composition of grapes was investigated to allow evaluation of the wine color and phenolic potential of the grapes. The feasibility of using mid infrared spectroscopy for the measurement of the phenolic composition of grapes and wines was also investigated and this examination included the development of a fast protocol for extraction of phenolics from grapes.

For the development of a fast extraction protocol, the influence of several factors on the extraction degree of phenols from red grapes by solvent extraction was investigated. Extraction temperature and solvent levels of both ethanol and hydrochloric acid exerted highly significant effects on the extraction of total phenols and anthocyanins from homogenates of red grapes. From these results an optimal extraction procedure was developed, which extracted a high percentage of both total phenols (81.8 %) and anthocyanins (91.5 %) with only a short solvent contact time (5 minutes). The extraction protocol was tested on eight different grape cultivars and found to produce consistent results across the different cultivars. Based on these results, the protocol was concluded to be a suitable grape extraction method to be employed in the assessment of the prediction of color attributes of wines from analyses of grape phenols.

The relation between the phenolic compositions of grapes and the corresponding red wines was then investigated for the developed grape extraction protocol and wines produced by microvinification. The proportion of wine phenols recovered from the grapes varied considerably for the different phenolic classes, with average ratios between 0.25 and 7.9. The average ratios for anthocyanins (0.31), total phenols (0.44), and tannins (0.32) were in accordance with the available knowledge pertaining to industrial winemaking, namely that even with prolonged maceration, the extraction of polyphenols rarely exceeds 50% of the total grape phenolic content (Haslam 2005). The variations in the recovery values for the different phenolic classes were concluded to be a result of varying extraction kinetics and chemical transformations taking place during winemaking.

Good direct relationships ($r \geq 0.88$) between the grape and wine phenols were observed for anthocyanins, total phenols, (+)-epicatechin, and (+)-catechin, while the direct relationships for the other phenolic classes were less evident. A multivariate approach to predict the phenolic composition of wines from the detailed phenolic composition of grapes only gave minor improvements, compared to the direct relations. In particular polymeric pigments were predicted more accurately using a multivariate approach ($r = 0.91$) than the direct relation between the grape and wine polymeric pigments ($r = 0.78$). The more accurate prediction was mainly ascribed to a good direct correlation between grape anthocyanins and wine polymeric pigments ($r = 0.87$), and it was concluded that a multivariate approach was able to account for such correlations.

The study also established that many wine color attributes of the wines (pH normalized) correlated particularly well with the levels of anthocyanins in the grapes. Although the multivariate modeling based on the detailed phenolic composition of the grapes allowed a slightly better prediction of the color attributes, it was in fact

sufficient to use only the grape anthocyanins for the color predictions. From the anthocyanin levels in grapes it was thus concluded that it was possible to predict several color attributes of the pH normalized red wines. The predicted color attributes from grape anthocyanins were: color intensity, Boulton's color values (except color due to polymeric pigments), and the CIELab color values L*, b*, and C*. The predictive residual deviation (RPD) values for these color attributes were between 2.4 and 5.7. Values above 3.1 are recommended to be good enough to allow a "fair" prediction (Williams 2001) which is why it is concluded that the predictions are mainly recommendable for screening purposes.

The feasibility of FT-MIR spectroscopy for the measurement of the phenolic composition of grapes and wines was investigated. For commercial red wines it was found that the levels of tannins could be quantified with FT-MIR spectroscopy satisfactorily (RPD ~2.7) when important spectral regions were selected for the calibration. It was however not possible to quantify the less abundant phenolic classes (e.g. anthocyanins) in the commercial red wines. The poor quantification of anthocyanins in commercial red wines by FT-MIR was concluded to be a result of the low concentrations of the anthocyanins in wines and spectral interferences from other components.

The most abundant phenolic components in the young wines and grape extracts could be quantified with FT-MIR spectroscopy. In young wines, total phenols and tannins were quantified very well (RPD values > 5), while anthocyanins only to some extent could be quantified by FT-MIR spectroscopy (RPD values between 2.5 and 2.9). In grapes extracts, total phenols and tannins were quantified well (RPD values between 3.2 and 5.0), while anthocyanins were more difficult to measure and only to some extent were quantifiable (RPD values between 2.1 and 2.6). From the FT-MIR spectra of the grape extracts the following wine color attributes were predicted with RPD values between 2.4 and 4.3: Color intensity, Boulton's color values (except color due to copigmentation), and the CIELab color values L*, b*, and C*. Hence it was concluded that the FT-MIR spectra of the grape extracts to some extent allowed prediction of wine color attributes.

Practical implications and future perspectives

Although other phenolic compounds than anthocyanins are known to influence wine color, by e.g. copigmentation or formation of polymeric pigments, the evaluation of the potential of red grapes with regards to the wine color of young wines was to a large degree ascribed to the anthocyanin content of the grapes. Thus the measurement of the levels of anthocyanins in the grapes would be sufficient for the evaluation of the wine color potential of the grapes.

The results obtained by use of the extraction protocol developed in this work showed that it was possible to obtain a high extraction of anthocyanins from the grapes with a short solvent contact time. However quantification of anthocyanins in the grape extracts by FT-MIR spectroscopy was only possible to some extent and the data indicated that the results would mainly be useful for rough screening purposes. The use of grape anthocyanins determined by FT-MIR spectroscopy can therefore only be recommended as supportive information in conjunction with other evaluations for decisions regarding payment purposes, harvesting decisions, segregations, and

processing conditions. Further development on measurements of grape anthocyanins should focus on improving the accuracy of the spectroscopic measurements.

The current best solution for quantification of grape anthocyanins is probably the NIR/VIS reflectance spectroscopy (Gishen et al. 2005, Janik et al. 2007), which also does not require a labor intensive extraction step. NIR/VIS (Cozzolino et al. 2004) and UV/VIS spectroscopy (Skogerson et al. 2007) has also showed good results for measuring different phenolic classes in fermenting must and wines. The use of spectroscopic methods in the visible region therefore seems to be a good technique for determining phenolic components having colored properties. Recently, the Winescan^{TM} instrument was updated to include measurements in the visible regions and such measurements could be suitable for the measurement of anthocyanins and other phenolic pigments. An investigation of the feasibility of using measurements in the visible range for quantification of anthocyanins would be an important next step.

In general the best calibration models for phenols were developed for tannins and total phenols. However, the correlation between the tannins levels in grapes and wines were poor, and therefore it is difficult to predict wine tannins from grape measurements. This problem could for instance be addressed by developing a protocol for grape extractions that is more representative to tannin extractions during winemaking. Another approach would be to use FT-MIR spectroscopy to monitor tannin concentration in the fermenting wine during maceration to ensure an appropriate tannin extraction. Finally tannin measurements by FT-MIR spectroscopy may also find applications as quality control tools within wine blending operations, where a certain wine style is desirable. Applications of systematic tannin measurements by FT-MIR spectroscopy may pave the way for the provision of a deeper understanding of the role of tannins in wine color and wine quality.

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CHAPTER 6 ABBREVIATIONS

a*	Degree of redness/greenness
abs	Absorbance
ANOVA	Analysis of variance
Anth-HPLC	Anthocyanins by HPLC
Anth-spec	Anthocyanins by spectroscopy
ATR	Attenuated total reflection
b*	Degree of blueness/yellowness
bi-PLS	Backward interval PLS
C*	Chroma
CE	Catechin equivalents
CFAE	Caffeic acid equivalents
CIE	Commission International L'Eclairage
cv	Cross validation
EC	Enzyme commission
EtOH	Ethanol
FT-MIR	Fourier-transform mid infrared
GA-PLS	Genetic algorithm PLS
Glc	Glucose
H*	Hue angle
HPLC	High performance liquid chromatography
IBECSI-PLS	Iterative backward elimination changeable size interval PLS
IR	Infrared
L*	Lightness
LPP	Large polymeric pigments
LV	Latent variables
Me	Methyl
ME	Malvidin-3-glucoside equivalents
MIR	Mid infrared
MP	Monomeric pigments
NIR	Near infrared
O.I.V.	Office Internationale de la Vigne et du Vin
PC	Principal component
PCA	Principal component analysis
PLS	Partial least squares
PP	Polymeric pigments
pred	Prediction
r	Correlation coefficient
R.T.	Room temperature
rel SD	Relative standard deviation

RMSECV	Root mean square error of cross validation
RMSEP	Root mean square error of prediction
RPD	Residual predictive deviation
RUE	Rutin equivalents
SD	Standard deviation
SECV	Standard error of cross validation
SEP	Standard error of prediction
si-PLS	Synergy interval PLS
SPP	Small polymeric pigments
Temp	Temperature
TFA	Trifluoroacetic acid
UV	Ultraviolet
VIS	Visible
WC anth	Wine color due to anthocyanins
WC copig	Wine color due to copigmentation
WC pp	Wine color due to polymeric pigments

CHAPTER 7 PUBLICATIONS

Paper I
Jensen, J.S., Blachez, B., Egebo, M., and Meyer, A.S. 2007. Rapid Extraction of Polyphenols from Red Grapes. Am. J. Enol. Vitic. 58:451-461.

Paper II
Jensen, J.S., Werge, H.H.M., Egebo, M., and Meyer, A.S. 2008. Effect of Wine Dilution on the Reliability of Tannin Analysis by Protein Precipitation. Am. J. Enol. Vitic. 59:103-105.

Paper III
Jensen, J.S., Demiray, S., Egebo, M., and Meyer, A.S. 2008. Prediction of Wine Color Attributes from the Phenolic Profiles of Red Grapes (*Vitis vinifera*). J. Agric. Food Chem. 56:1105-1115.

Paper IV
Jensen, J.S., Egebo, M., and Meyer, A.S. 2008. Identification of Spectral Regions for Quantification of Red Wine Tannins with Fourier Transform Mid-Infrared Spectroscopy. J. Agric. Food Chem. Accepted for publication.

Paper I

Jensen, J.S., Blachez, B., Egebo, M., and Meyer, A.S. 2007. Rapid Extraction of Polyphenols from Red Grapes. Am. J. Enol. Vitic. 58:451-461.

Rapid Extraction of Polyphenols from Red Grapes

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Abstract: Assessment of the phenolic content of red grapes is an important prerequisite for understanding how grape phenols impact wine quality. The influence of selected factors on extraction efficiency of phenols from eight different red grape cultivars was investigated to determine a rapid and robust extraction method. The effects of solvent contact time, extraction temperature, and concentration of ethanol and hydrochloric acid on extraction of total phenols and anthocyanins were investigated in a series of statistically designed factorial experiments. The results were compared to the "total" concentrations as measured by a modified "total" extraction protocol and the data were expressed as relative extraction efficiencies. Both extraction temperature and concentration of the solvents ethanol and hydrochloric acid exerted highly significant effects on the extraction of both total phenols and anthocyanins. The optimized extraction procedure was as follows: mix 50% v/v acidified aqueous ethanol (0.1 M HCl) in a 1:1 v/w ratio with crushed grapes to give a final ethanol of ~25% v/v for 5 min at 40ºC, followed by neutralization and clarification. By this rapid procedure, it was possible to extract an average 81.8% of the total phenols and 91.5% of the anthocyanins from the grapes.

Key words: solvent extraction, red grapes, anthocyanins, total phenols

Red wine color is mainly dependent on the content and composition of the phenolic substances present, including notably the total concentration of anthocyanins and polymeric pigments (Bakker et al. 1986, Mazza et al. 1999, Sacchi et al. 2005). Although anthocyanins dominate the color of young red wines, other substances such as tannins play a major role in long-term wine color development and, hence, influence the wine color stability during aging (Cheynier et al. 2006). The color forms an important part of the perceived quality of red wine (Somers and Evans 1974), but the phenolic compounds also affect aroma and mouthfeel (Preys et al. 2006). Several changes in the phenolics occur during red wine production, particularly during fermentation and maturation. Nevertheless, a main hypothesis in our ongoing work on grape and wine phenols is that the phenolics present in the grapes significantly influence the quality of the finished wine and that it may be possible to predict wine quality from analysis of the phenolics in grapes.

A first step in testing this hypothesis is to establish a relationship between the phenolics in grapes and those in the resulting red wines. However, since assessment of the content and profile of phenolics present in grapes is strongly dependent on the extraction method employed, a prerequisite for obtaining a proper evaluation of the phenolics present in grapes is to define a robust extraction method for grape phenols.

Condensed tannins and anthocyanins are the two most abundant classes of polyphenols found in grapes. After the onset of veraison, anthocyanins accumulate in the skins while condensed tannins of both seeds and skin decrease during ripening (Adams 2006, Kennedy et al. 2000). Anthocyanins are easily extracted from grape skins with different solvents (acidic methanol, acidic ethanol, or acetone/water) that are also the conventionally most widely used solvents for phenols extraction from grapes (Macheix et al. 1990). The extraction of phenols from the seeds is more challenging. The seeds are generally not crushed during winemaking and the extraction of gallic acid, condensed tannins, and catechins from intact seeds into the fermenting wine is only achieved slowly during 5 to 12 days of maceration with a gradually increasing ethanol concentration that facilitates extraction from the seeds and skins (Gonzalez-Manzano et al. 2004).

The majority of seed polyphenols are located in the outer seed coat (Thorngate and Singleton 1994), and it is possible to extract the majority of the seed polyphenols under simulated but realistic winemaking conditions (Singleton and Draper 1964). However, when grape seeds are left intact during rapid extraction for phenols analysis, little or no phenols are extracted from the seeds (Meyer et al. 1997). In contrast, when the grape seeds are crushed, flavan-3-ols and gallic acid are rapidly extracted from the seed tissue. Hence, crushing of the grape seeds is required to obtain a representative extraction of phenols from the seeds during rapid grape extraction.

A complete extraction method for grape anthocyanins and total phenols, which requires a solvent contact time of 1 hour and a high ratio (10:1 v/w) of solvent to sol-

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ids, has been reported (Iland et al. 2004). Other reported methods also rely on long extraction times, use of different organic solvents, or tedious, multistep sample preparation (Kallithraka et al. 1995, Saint-Cricq de Gaulejac et al. 1998). Surprisingly few studies have systematically addressed how different parameters of the grape phenols extraction process such as temperature, time, solvent-acidification, or type of cultivar quantitatively affect extraction of phenols from grapes intended for wine production.

In the context of establishing a consistent, rapid method for obtaining high extraction efficiencies of phenols from grapes, the objective of this study was to evaluate the influence of selected parameters expected to affect extraction efficiency and robustness and to identify an optimal extraction procedure. To mimic the events taking place during winemaking and avoid use of potentially toxic, highly volatile, or flammable organic solvents, ethanol was chosen as the most relevant extraction solvent for the study. The effects of extraction temperature, addition of acid, ethanol concentration, and extraction time (that is, solvent contact time) were evaluated on eight different red grape cultivars in statistically designed experiments using total phenols and anthocyanins as responses.

Materials and Methods

Grape samples and chemicals. Grape samples of eight different red cultivars (Alicante, Merlot, Syrah, Cinsault, Grenache, Carignan, Cabernet Sauvignon, and Mourvedre) of *Vitis vinifera* were collected in the south of France in August and September 2005. Mature grapes were manually picked from vines from the same field location, frozen in polyethylene bags, and stored at -30ºC until use. Technical grade 96% v/v ethanol (V&S Distillers, Aalborg, Denmark) and analytical grade hydrochloric acid (Merck, Darmstadt, Germany) were used to prepare extraction solvents. HPLC-grade acetonitrile, *o*-phosphoric acid, gallic acid, (+)-catechin hydrate, (-)-epicatechin, rutin hydrate, caffeic acid, and malvidin-3-glucoside hydrochloride for HPLC solvents and standards were purchased from Sigma-Aldrich (St. Louis, MO). Chemicals for tannin analysis (bovine serum albumin [BSA, fraction V powder], tartaric acid, sodium dodecyl sulfate [SDS], acetic acid, sodium chloride, triethanolamine [TEA], and ferric chloride hexahydrate) were all of analytical grade and also purchased from Sigma-Aldrich.

Grape homogenization. Grapes were manually destemmed while frozen, placed in polyethylene bags, and sample aliquots for each extraction experiment were gently thawed in a water bath at room temperature for approximately 1 hour. The grapes were then crushed with an Ultra-Turrax T25 high speed homogenizer (IKA-Werke & Co. GmbH KG, Janke & Kunkel, Staufen, Germany) at 24,000 rpm under a stream of nitrogen for a defined time (i.e., 0.5 to 4 min), according to the experimental plan. The resulting grape purée of juice, skin, seeds, and pulp is referred to as grape homogenate in the following.

Estimation of juice content. 50 g grape homogenate was weighed and centrifuged (20 min, 4,800 *g*) and the resulting juice was passed through Whatman grade 4 filter paper (Whatman International, Kent, UK). For each sample, the juice density was estimated by weighing a 25-mL sample. The remaining solids were rinsed with 25 mL water, centrifuged 20 min at 4,800 *g*, filtered, and dried at 105ºC overnight to give the mass of insoluble solids. The juice content was expressed as mL juice/g grape and the values obtained were used to calculate the concentrations of anthocyanins and total phenols/g grape.

Total extraction protocol. For benchmarking, an estimate of "total" anthocyanins and phenols present in the different grape samples was made via a modified version of an existing "total" extraction protocol (Iland et al. 2004). The modification was that the solid grape residues from the first extraction were subjected to a second extraction, so that the final extraction protocol was as follows: 2 g grape homogenate was weighed and extracted for 1 hour at room temperature (~25ºC) with 20 mL aqueous ethanol (50% v/v, adjusted to pH 2 with HCl). During the solvent contact, the mixture was continuously mixed on an oscillating inverter. The extract was then centrifuged 5 min at 16,100 *g* and diluted 21 times with 1 M HCl. The resulting solid residues were rinsed with distilled water, recovered by filtration through Whatman grade 4 filter paper, and re-extracted overnight with 20 mL aqueous ethanol at room temperature during continuous mixing. The second extract was centrifuged 5 min at 16,100 *g* and diluted 7.67 times in 1 M HCl (the dilutions were made to target the optimal range for subsequent absorbance measurements). The absorbances at 280, 520, and 700 nm of the diluted samples, referred to as abs(280 nm), abs(520 nm), and abs(700 nm), respectively, were measured in 10-mm quartz cuvettes on a spectrophotometer (Cary 300, Varian, St. Helens, Australia). The abs(700 nm) data were used to evaluate the turbidity of the samples. The anthocyanin concentration was expressed as mg malvidin-3-glucoside equivalents/g grape from the absorbance at 520 nm via use of a generic extinction coefficient $\varepsilon = 50$ mL/(mg · cm) (equation 1) (Iland et al. 2004, Somers and Evans 1977). Total phenols were expressed as 0.01 absorbance units at 280 nm/g grape (equation 2) (Iland et al. 2004).

$$\text{Anthocyanins (mg/g)} = (V + m \cdot \text{juice content}) \cdot DF \cdot \text{abs}(520\text{ nm}) / (m \cdot \varepsilon) \quad \text{(eq 1)}$$

$$\text{Total phenols (0.01 abs/g)} = (V + m \cdot \text{juice content}) \cdot DF \cdot \text{abs}(280\text{ nm}) / (100 \cdot m) \quad \text{(eq 2)}$$

V is the volume of the added extraction solvent in mL, m is the mass of the extracted grape homogenate in g, juice content is the volume of juice in mL/g grape, DF is the dilution factor of the extract in 1 M HCl, and ε is the generic extinction coefficient of malvidin-3-glucoside in mL/(mg · cm). In the calculations, it was assumed that the material used for the re-extraction did not contain any

juice (juice content = 0 mL/g). The total concentrations of anthocyanins and total phenols, respectively, were then calculated as the sum of the first and second extraction yields. For each grape cultivar, the benchmark "total" phenols and anthocyanins were determined from at least duplicate extractions.

Experiment 1. Fast extraction of phenols and anthocyanins. The effect of extraction temperature (20, 40, or 60ºC), solvent composition (0, 25, and 50% v/v ethanol), and hydrochloric acid concentration (0 or 0.1 M) on yields of total phenols and anthocyanins was examined in a randomized, full factorial design with one determination on each factor combination and three center points. For all extractions, a 1:1 weight:volume ratio of grape homogenate:solvent was used, as this ratio resulted in maximum phenols extraction in preliminary experiments (data not shown). For each extraction experiment, 400 g grapes were homogenized for 2.5 min and immediately thereafter aliquots of 15 g homogenate were transferred into individual beakers containing a stirring magnet. Solvent was added according to the experimental plan and the samples were sealed, placed in a magnetic stirring water bath, and incubated with stirring at defined temperatures of 20, 40, or 60ºC for 30 min according to the experimental plan. After the solvent contact period, 2-mL aliquots of each sample were centrifuged for 5 min at 16,100 *g* and diluted 101 times in 1 M HCl. The different dilution factors for total and fast extractions were necessary because of the inherently higher solvent to solid ratio used in the total extraction protocol.

After 1 hour, the absorbances of the diluted samples were read at 280, 520, and 700 nm using 10-mm quartz cuvettes and the amount of anthocyanins and total phenols for both the fast solvent extractions and total extractions were calculated from equations 1 and 2. The abs(700 nm) data were used to evaluate sample turbidity. Each individual extraction yield was calculated as a relative percentage of the total determined on the same homogenate in triplicate using the total extraction protocol (Figure 1). The experiment was repeated for nine samples, covering the different grape cultivars under investigation: Alicante, Merlot (two different samples), Syrah, Cinsault, Grenache, Carignan, Cabernet Sauvignon, and Mourvedre (Table 1).

Effect of homogenization and solvent contact times. 180 g grapes (Alicante only) were destemmed, thawed, and homogenized as described above. During homogenization, 15-g samples were taken after 0.5, 1, 3, and 4 min of homogenization (single run). Each sample was supplemented with 15 mL 25% aqueous ethanol containing 0.1 M HCl, sealed, and stirred with a magnet on a 40ºC water bath. A 2-mL sample was subsequently withdrawn from each beaker containing ~30 g after 0, 2, 5, 15, and 30 min, centrifuged 1 min at 16,100 *g*, and diluted 51 times in 1 M HCl. The absorbances of the diluted samples were then measured at 280, 520, and 700 nm after 1 hour. The concentrations of anthocyanins and total phenols were calculated from equations 1 and 2 and the results were expressed as relative percentages of the anthocyanins and total phenols obtained in triplicate with the modified extraction protocol. The abs(700 nm) data were used to evaluate the turbidity of samples.

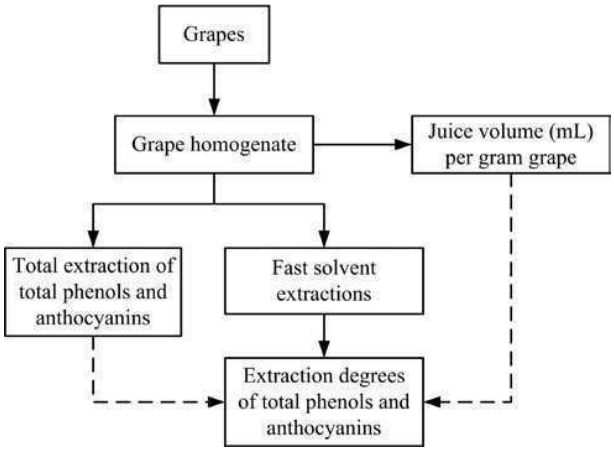

Figure 1 Experimental overview.

Table 1 Red grapes used for extraction of polyphenols.

Cultivar	Exp 1	Exp 2	Exp 3
Alicante 1	x		
Alicante 2		x	x
Cabernet Sauvignon 1	x	x	x
Carignan 1	x		
Carignan 2		x	x
Cinsault 1	x		
Cinsault 2		x	x
Grenache noir 1	x		
Grenache noir 2		x	x
Merlot 1	x	x	x
Merlot 2	x		
Mourvedre 1	x	x	x
Syrah 1	x		
Syrah 2		x	x

Experiment 2. Time-optimized extraction conditions. 150 g grapes were destemmed, thawed, and homogenized for 2 min as described above. Homogenate portions (16 g) were then transferred quickly to four beakers. Each sample was mixed with 1 mL 1.6 M HCl and 15 mL aqueous ethanol (53.3% v/v) that had been preheated to 60ºC to minimize the heating time of the grape sample. These mixtures were then sealed and stirred with magnetic stirring at 40ºC. After 5 and 15 min, respectively, the extracts (each in duplicate) were centrifuged for 5 min at 16,100 *g* and diluted 51 times in 1 M HCl. After 1 hour, the absorbances of these acidified samples were read at 280, 520, and 700 nm in 10-mm quartz cuvettes. The amounts of anthocyanins and total phenols were calculated from equations 1 and 2 and the results were expressed as relative percentages of the anthocyanins and total phenols obtained in duplicate with the total extrac-

tion protocol. To test the consistency of extraction among cultivars, the extractions were carried out on Alicante, Merlot, Syrah, Cinsault, Grenache, Carignan, Cabernet Sauvignon, and Mourvedre grapes (Table 1).

Experiment 3. Final extraction protocol. 150 g grapes were destemmed, thawed, and homogenized for 2 min as described above. 50 g homogenate was weighed in a glass bottle and mixed with 50 mL acidic (0.1 M HCl) aqueous ethanol (50% v/v), which had been preheated to 60ºC. The mixture was sealed and then stirred vigorously with a magnetic bar at 40ºC. After 5 min, 1 mL 5 M sodium hydroxide solution was slowly added while stirring for 1 min to neutralize the added hydrochloric acid. The sample was centrifuged 10 min at 15,000 *g* and filtered through a Whatman grade 4 cellulose filter. Finally, the extracts were centrifuged for 5 min at 23,000 *g* and diluted 101 times in 1 M HCl. After 1 hour, the absorbances of these acidified samples were read at 280, 520, and 700 nm in 10-mm quartz cuvettes. The concentrations of anthocyanins and total phenols were calculated from equations 1 and 2 and the results were expressed as relative percentages of the anthocyanins and total phenols obtained in duplicate with the total extraction protocol. The abs(700 nm) data were used to evaluate the turbidity of the samples. To test the consistency of extraction among cultivars, the extractions were carried out on Alicante, Merlot, Syrah, Cinsault, Grenache, Carignan, Cabernet Sauvignon, and Mourvedre grapes (Table 1).

Stability of phenolics during prolonged solvent contact. 100 g Merlot grapes were destemmed, thawed, and homogenized as described above. The homogenate (50 g) was extracted with 50 mL 50% v/v EtOH at ambient temperature by stirring for 1 hour, centrifuged for 10 min at 15,000 *g*, filtered through a Whatman grade 1 filter paper, further centrifuged for 10 min at 23,000 *g,* and finally filtered through a Titan2 0.45-µm nylon syringe filter (Sun Sri, Rockwood, TN) to obtain a solution that was practically free of particles. Two 10-mL aliquots were mixed with 10 mL extraction solvent (25% v/v ethanol, 0.1 M HCl, preheated to 60ºC) and stirred at 40ºC for 5 and 30 min, respectively. Immediately after the solvent contact period, the samples were neutralized with 0.2 mL 5 M sodium hydroxide and the volumes were adjusted to 25 mL with water. For comparison, an untreated sample was prepared by diluting a 10-mL aliquot to 25 mL with water. All samples were flushed with nitrogen and frozen for later phenolic analysis by HPLC and protein precipitation.

Analysis of phenolic compounds by HPLC. Samples from the stability study were analyzed on an Agilent 1100 series HPLC instrument (Agilent, Waldbronn, Germany) equipped with a vacuum degasser, a quaternary pump, an autosampler, a thermostatted column compartment, and a diode array detector as described (Lamuela-Raventos and Waterhouse 1994), with some modifications. A Gemini C18 column (150 mm x 4.6 mm, 3-µm particle size, 110 Å pore size) (Phenomenex, Torrance, CA) with a 4 x 3 mm guard column of the same material was used as stationary phase at 40ºC. The solvents were: solvent A (water with 0.20 M *o*-phosphoric acid and 3% v/v acetonitrile, adjusted to pH 1.50 with aqueous sodium hydroxide) and solvent B (a 1:1 v/v mixture of solvent A and acetonitrile). A constant flow of 0.5 mL/min was applied with a linear gradient elution profile of 0 min (11% solvent B), 40 min (40% solvent B), 50 min (60% solvent B), 53 min (100% solvent B), 60 min (100% solvent B), 61 min (11% solvent B), and 65 min (11% solvent B). Prior to injection, each sample was centrifuged at 23,000 *g* for 5 min, filtered through a Phenex 0.45-µm nylon syringe filter (Phenomenex), and stored under nitrogen until analysis. The injection volume was 10 µL. The compounds were identified according to their retention times and spectral properties. Gallic acid, (+)-catechin, and (-)-epicatechin were quantified at 280 nm from external standard curves of authentic standards. Hydroxycinnamates were collectively estimated at 316 nm (for peaks having absorption maxima at 316 nm) and expressed as mg caffeic acid equivalents/L by comparison with an external standard curve of caffeic acid. Flavonols were collectively estimated at 365 nm (for peaks having characteristic absorption maxima at 365 nm) and expressed as mg rutin equivalents/L by comparison with an external standard curve of rutin hydrate. Anthocyanins were quantified at 520 nm and expressed as mg malvidin-3-glucoside equivalents/L by comparison with a standard curve of malvidin-3-glucoside hydrochloride.

Analysis of tannins by protein precipitation. Samples from the stability study were analyzed by the precipitation method as described (Harbertson et al. 2003) with a few modifications due to equipment limitations. Prior to analysis, the samples were diluted in a model wine solution of 12% v/v ethanol with 5 g/L tartaric acid, which had been adjusted to pH 3.3 with sodium hydroxide. The tannin-protein precipitate was formed by mixing 0.5 mL diluted wine and 1 mL BSA solution (containing 1 mg BSA/mL dissolved in a buffer of aqueous 0.2 M acetic acid and 0.17 M sodium chloride adjusted to pH 4.9) for 30 min. The precipitate was centrifuged at 14,000 *g* for 5 min to form a pellet and the supernatant was discarded. The pellet was washed with 0.25 mL of the pH 4.9 buffer, centrifuged at 14,000 *g* for 3 min, and the supernatant was discarded. The washing step was repeated one time. The rinsed pellet was redissolved in 1.5 mL buffer containing 5% w/v sodium dodecyl sulfate and 5% v/v triethanolamine by gentle mixing for 20 min. The background was measured as the absorbance at 510 nm of 1 mL redissolved solution. The sample was then mixed with 125 µL 11.4 mM ferric chloride in 11.4 mM aqueous HCl, and final absorbance at 510 nm was recorded after 10 min. The tannin was calculated as 1.125 times the final absorbance minus the background absorbance. The tannin concentration was expressed as mg catechin equivalents (CE)/L from a standard curve of the color reaction between catechin and ferric chloride.

Statistical data analysis. The full factorial designs in experiment 1 were fitted to a linear model accounting for main and interaction effects using SAS JMP software (version 5.1, SAS Institute, Cary, NC). The response levels y_i for all i observations were estimated in a linear model of the three factor levels (x_1, x_2, and x_3) accounting for main and interaction effects (equation 3):

$$y_i = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \varepsilon_i \quad \text{(eq 3)}$$

The best linear model was then found by multiple linear regression to minimize the sum of squares of the residual values ε_i by the method of least squares (Montgomery 2001). The effect of each factor combination was estimated using mean-centered factor levels scaled between -1 and 1 to allow direct comparison of β values. Significances of differences were accepted on a 95% confidence level ($p < 0.05$).

Results and Discussion

The published "total" extraction protocol includes one extraction step only (Iland et al. 2004). To establish the benchmark for assessing the extent of phenols extraction from the different grape cultivars, the yields of total phenols and anthocyanins obtained in individual extraction steps were evaluated when repeating the "total" extraction procedure. The data obtained showed that almost all anthocyanins were removed during the first extraction, as the average yields of the second extraction on the different grape varieties were less than 3% of the sum of the first and second extractions (Table 2). The yields of the third and fourth extractions were essentially nothing (data not shown). In contrast, 3.8 to 14.4% of the total phenols (the percentages calculated from the sum of the first and second extraction) were removed in the second extraction. For this reason, we decided to modify the published method by using the sum of the first and second extraction of the anthocyanins and total phenols as the benchmark "total."

Experiment 1. Rapid solvent extraction of phenols and anthocyanins. The mean extraction efficiency from the factorial design experiment evaluating extraction temperature (20, 40, or 60ºC), ethanol concentration (0, 25, or 50% v/v), and hydrochloric acid concentration (0 or 0.1 M) ranged from 33 to 99% for total phenols and from 42 to 100% for anthocyanins (Table 3). The relative standard deviations across the nine samples ranged from 4.7 to 17.1% for total phenols and from 1.7 to 8.6% for anthocyanins.

As expected, wide variations in the responses were recorded with the different extraction treatments. The three main factors of extraction temperature, ethanol concentration, and hydrochloric acid concentration exerted a significant influence on the extraction efficiency of both anthocyanins and total phenols ($p < 0.0001$) (Table 4). Comparison of the estimated β parameters signified that ethanol had the greatest influence on the extent of extraction achieved (β = 12.8 to 18.8), while the influence of hydrochloric acid (β = 6.7 to 8.0) and extraction temperature (β = 5.5 to 6.6) were of lower magnitude. No interaction effects were significant for total phenols, but for anthocyanins significantly negative interaction effects (β = -1.8 to -5.5) were recorded for all pairwise combinations. For total phenols, the increase in the extraction efficiency by acidifying the solvent was 10 to 15% irrespective of temperature and ethanol concentration (Figure 2A), while anthocyanin extraction increased between 5 and 35% with acidification (Figure 2B). Moreover, the extraction of anthocyanins appeared to be more affected by acidification at the lower ethanol concentrations and lower temperatures.

Table 2 Total extraction of phenols and anthocyanins in experiment 1.

Cultivar	Juice content (mL/g)	Total phenols (0.01 abs/g)[a] Mean (n = 3)	rel SD[c]	Re-extraction (%)[d]	Anthocyanins (mg/g)[b] Mean (n = 3)	rel SD[c]	Re-extraction (%)[d]
Alicante 1	0.86	2.85	2.8	3.8	4.15	4.0	1.7
Merlot 1	0.80	2.40	0.2	8.8	2.04	0.5	2.0
Merlot 2	0.87	1.43	1.0	11.9	1.45	2.0	2.6
Syrah 1	0.87	2.21	1.3	10.0	2.55	2.0	2.1
Cinsault 1	0.89	1.12	1.7	12.8	0.94	4.0	4.7
Grenache 1	0.89	1.29	1.0	14.1	0.82	0.9	4.4
Carignan 1	0.88	1.48	1.1	10.6	1.59	1.7	2.0
Cab. Sauv. 1	0.86	1.83	1.5	8.4	1.70	1.6	2.2
Mourvedre 1	0.87	1.71	1.7	14.4	1.24	1.8	3.8
Mean	0.87	1.81	1.4	10.5	1.83	2.1	2.8

[a]Total phenols expressed as 0.01 absorbance units/g grape.
[b]Anthocyanins expressed as mg malvidin-3-glucoside equivalents/g grape.
[c]The relative standard deviation in % from triplicate extractions of the homogenate.
[d]Re-extraction % is the percentage of the second extraction compared to the total content, defined as the sum of first and second extraction.

Table 3 Fast solvent extraction of total phenols and anthocyanin for the samples in Table 2.

			Extracted total phenols (%)		Extracted anthocyanins (%)	
EtOH (% v/v)	HCl (M)	Temp (°C)	Mean (n = 9)[a]	Rel SD[b]	Mean (n = 9)[a]	Rel SD[b]
0	0	20	33.8	12.4	42.2	8.6
25	0	20	53.9	11.8	70.0	5.1
50	0	20	73.0	11.2	88.9	4.2
0	0.1	20	49.2	17.1	77.0	6.0
25	0.1	20	66.9	11.4	88.7	2.8
50	0.1	20	82.7	8.1	94.4	3.0
0	0	40	40.7	12.3	55.7	7.8
25	0	40	62.2	13.4	80.4	6.0
50	0	40	78.7	11.1	90.4	5.9
0	0.1	40	54.0	16.8	83.6	5.6
25	0.1	40	74.9	11.0	93.1	2.6
50	0.1	40	91.7	6.5	97.6	2.9
0	0	60	46.0	13.1	65.3	6.7
25	0	60	68.2	14.0	85.5	6.5
50	0	60	83.6	10.7	94.0	3.6
0	0.1	60	59.7	13.4	87.8	1.7
25	0.1	60	82.8	9.3	94.5	2.2
50	0.1	60	98.7	4.7	99.9	3.7
25	0.05	40	65.5	9.7	87.9	2.9
25	0.05	40	65.0	9.8	87.4	3.7
25	0.05	40	65.0	9.8	87.1	2.4
Mean			66.5	11.3	83.4	4.5

[a]Mean extraction efficiencies for the nine samples, calculated as % of the total amount.
[b]Relative standard deviation across the nine samples in %.

Table 4 Effect tests and estimated model parameters for the mean extraction efficiency (%) of total phenols and anthocyanins from experiment 1.

	Total phenols (model fit: R^2 = 0.99)			Anthocyanins (model fit: R^2 = 0.96)		
Term	Prob > F[a]	ß estimate[b]	SE	Prob > F[a]	ß estimate[b]	SE
Intercept	<0.0001	66.49	0.36	<0.0001	83.40	0.76
EtOH	<0.0001	18.75	0.48	<0.0001	12.80	1.00
HCl	<0.0001	6.69	0.39	<0.0001	8.00	0.82
Temp	<0.0001	6.63	0.48	<0.0001	5.48	1.00
EtOH*HCl	0.446	-0.37	0.48	<0.0001	-5.54	1.00
EtOH*Temp	0.425	0.48	0.58	0.032	-2.92	1.22
HCl*Temp	0.367	0.45	0.48	0.093	-1.80	1.00

[a]Prob > F describes the probability that a term does not have a significant effect.
[b]The ß estimates of the linear model using mean centered factor levels scaled between -1 and +1.

During one-way analysis of variance (ANOVA), mean relative standard deviations were significantly lower for anthocyanins than for total phenols ($p < 0.0001$). There was also a significant, negative correlation between mean extraction efficiency and relative standard deviation for anthocyanins (r = -0.730, $p < 0.0001$) and total phenols (r = -0.801, $p < 0.0001$) (Figure 3). However, the correlation for anthocyanins was not significant for samples in which >80% of the anthocyanins were extracted. These findings confirm that to make the method robust, a desirable protocol yields high extraction efficiency, especially with respect to total phenols. The factor combinations with 50% ethanol and 0.1 M hydrochloric acid at 40 and 60ºC showed high extraction efficiency and low variation across the different samples: at 40ºC, 91.7% total phenols (rel SD 6.5%) and 97.6% anthocyanins (rel SD 2.9%), and at 60ºC, 98.7% total phenols (rel SD 4.7%) and 99.9% anthocyanins (rel SD 3.7%). These two treatments therefore have good potential to meet the dual needs of high extraction efficiency and a robust extraction method.

Effect of solvent contact time. The effect of solvent contact time (15, 30, and 150 min) was tested for Alicante

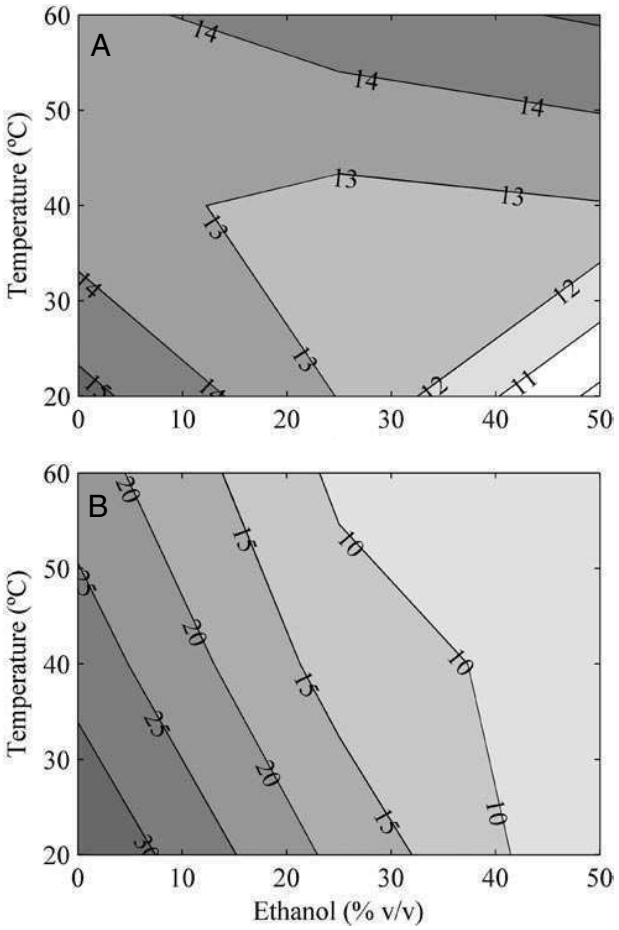

Figure 2 Contour plot of the increase in extraction efficiency (%) of (**A**) total phenols and (**B**) anthocyanins arising from solvent acidification from 0 to 0.1 M HCl. Numbers specify the increase in extraction efficiency due to acidification.

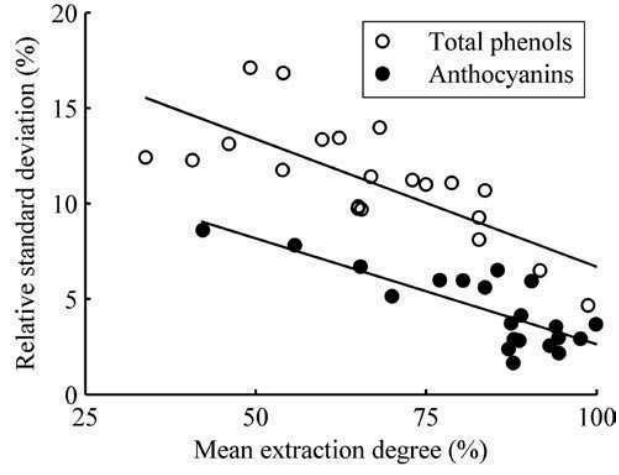

Figure 3 Correlation between extraction efficiency (%) and relative standard deviation (%) for extraction of total phenols and anthocyanins.

grapes. No significant difference in extraction after 15 and 30 min could be established for either total phenols ($p = 0.546$) or anthocyanins ($p = 0.483$), but for 15, 30, and 150 min there was a significant, negative effect of

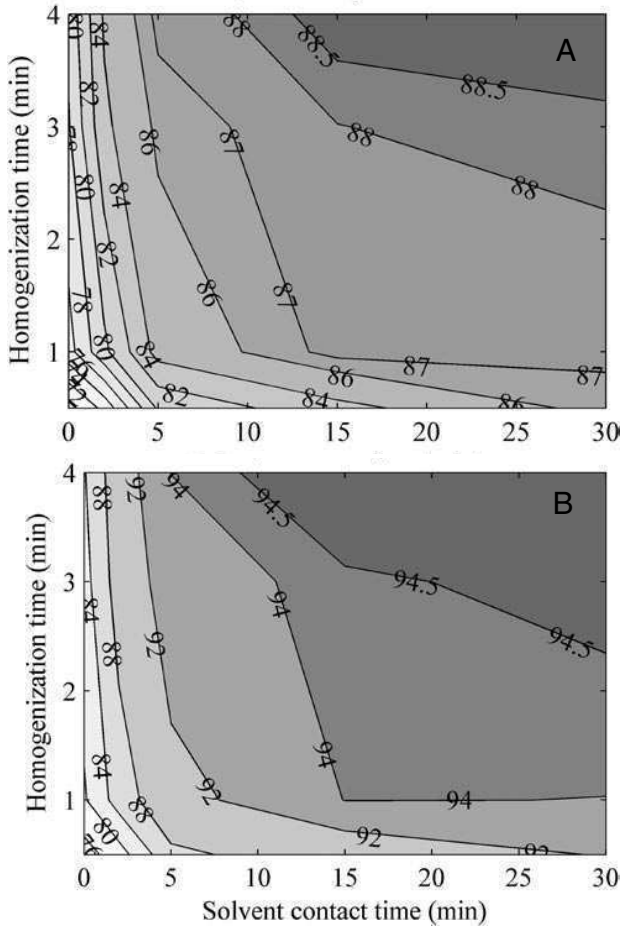

Figure 4 Contour plot of the influence of homogenization time and solvent contact time on the extraction efficiencies of (**A**) total phenols and (**B**) anthocyanins. Numbers specify extraction efficiency.

solvent contact time on both total phenols ($p < 0.0001$) and anthocyanins ($p < 0.0001$). In a supplementary experiment on Alicante grapes, we found a significantly negative effect of further increasing hydrochloric acid in the solvent from 0.1 M to 0.2, 0.5, or 1 M on the extraction of total phenols, but no significant effect on extraction of anthocyanins (data not shown).

Effect of homogenization time and solvent contact time. Since short extraction time was one goal of this research, the potential for using even shorter solvent contact time for extraction of phenols was further investigated. A full factorial design varying the homogenization time (0.5, 1, 3, and 4 min) and the solvent contact time (0, 2, 5, 15, and 30 min) on a single cultivar (Alicante) was carried out using constant conditions of 40ºC, 25% v/v ethanol, and 0.1 M hydrochloric acid.

The data from this factorial design were difficult to fit in a linear model accounting for both main and interaction effects. The R^2(total phenols) was 0.67 and the R^2(anthocyanins) was 0.61, likely due to a nonlinear response at low solvent contact times. Due to the imprecision of the linear models, responses were configured as contour plots (Figure 4). These plots clearly show that,

Table 5 Extraction efficiencies of total phenols and anthocyanins obtained by optimized fast experimental extraction (experiment 2) relative to the total extract benchmark results: total phenols (0.01 abs/g) and anthocyanins (mg/g).

				Extraction efficiency total phenols (%)				
		Total phenols (0.01 abs/g)[a]		5 min extraction		15 min extraction		
Cultivar	Juice content (mL/g)	Mean (n = 2)	rel SD (%)[c]	Mean (n = 2)	rel SD (%)[c]	Mean (n = 2)	rel SD (%)[c]	Grape[d] homogenate
Alicante 2	0.88	2.36	0.5	97.9	0.1	99.8	0.0	45.7
Merlot 1	0.84	1.65	0.3	86.7	1.0	91.9	1.0	33.5
Syrah 2	0.87	2.04	0.1	90.0	1.3	90.7	2.2	30.5
Cinsault 1	0.90	1.00	5.0	99.1	0.1	101.3	0.4	28.5
Grenache noir 2	0.88	1.40	5.1	101.6	1.0	104.5	0.5	37.0
Carignan 2	0.88	2.28	4.5	94.5	5.3	96.4	2.7	42.0
Cab. Sauv. 1	0.86	2.06	1.3	89.0	0.5	91.6	0.3	34.3
Mourvedre 1	0.86	1.90	1.6	89.6	0.1	91.3	0.1	30.7
Mean[e]	0.87	1.84	2.2	93.5	1.2	95.9	0.9	35.3
rel SD[e]	2.2%	25.2%		5.9%		5.6%		17.0%

				Extraction efficiency anthocyanins (%)				
		Anthocyanins (mg/g)[b]		5 min extraction		15 min extraction		
	Juice content (mL/g)	Mean (n = 2)	rel SD (%)[c]	Mean (n = 2)	rel SD (%)[c]	Mean (n = 2)	rel SD (%)[c]	Grape[d] homogenate
Alicante 2	0.88	3.24	1.3	98.7	0.5	99.1	0.1	46.0
Merlot 1	0.84	1.80	0.6	95.2	0.3	95.5	1.0	42.0
Syrah 2	0.87	1.99	0.4	94.1	0.8	95.7	0.2	40.4
Cinsault 1	0.90	0.57	4.0	105.9	1.0	109.2	2.5	30.2
Grenache noir 2	0.88	0.97	7.0	105.4	1.8	107.5	0.3	42.4
Carignan 2	0.88	1.89	6.6	100.0	1.9	102.6	3.2	42.6
Cab. Sauv. 1	0.86	1.68	2.1	95.6	0.4	96.7	0.5	41.2
Mourvedre 1	0.86	1.68	2.2	96.1	0.1	97.9	0.6	38.7
Mean[e]	0.87	1.73	3.0	98.9	0.9	100.5	1.0	40.4
rel SD[e]	2.2%	45.3%		4.6%		5.3%		11.5%

[a]Total phenols expressed as 0.01 absorbance units/g grape.
[b]Anthocyanins expressed as mg malvidin-3-glucoside equivalents/g grape.
[c]Relative standard deviation in % from duplicate extractions of the homogenate.
[d]Extraction of the juice in the homogenate before the solvent extraction.
[e]Mean and relative standard deviations across the eight cultivars.

Table 6 Results of total extraction and fast extraction followed by sample neutralization of eight red grape cultivars.

		Total phenols (0.01 abs/g)[a]			Anthocyanins (mg/g)[b]		
Cultivar	Juice content (mL/g)	Total extraction (n = 3)	Fast extraction (n = 1)	Extraction efficiency (%)	Total extraction (n = 3)	Fast extraction (n = 1)	Extraction efficiency (%)
Alicante 2	0.89	2.16	1.92	88.9	2.81	2.58	91.8
Merlot 1	0.85	2.22	1.92	86.4	1.91	1.89	98.9
Syrah 2	0.88	1.77	1.44	81.6	1.79	1.56	87.1
Cinsault 1	0.88	1.03	0.84	81.8	0.68	0.63	91.8
Grenache noir 2	0.87	1.28	1.04	81.2	0.89	0.82	92.1
Carignan 2	0.89	1.43	1.17	81.7	1.52	1.39	91.3
Cab. Sauv. 1	0.85	1.86	1.51	80.9	1.56	1.41	90.4
Mourvedre 1	0.87	1.78	1.28	72.2	1.40	1.24	88.6
Mean[c]	0.87	1.69	1.39	81.8	1.57	1.44	91.5
rel SD[c]	1.8%	24.7%	28.0%	6.0%	41.5%	42.3%	3.8%

[a]Total phenols expressed as 0.01 absorbance units/g grape.
[b]Anthocyanins expressed as mg malvidin-3-glucoside equivalents/g grape.
[c]Mean and relative standard deviations across the eight cultivars.

under the given conditions, the major part of total phenols and anthocyanins are extracted within approximately the first 5 min of extraction and the first minute of homogenization.

Experiment 2. Time-optimized extraction conditions. The time-optimized extractions were conducted at 40ºC using preheated 50% v/v aqueous ethanol with 0.1 M hydrochloric acid and gave mean extraction efficiencies of 93.5% for total phenols and 98.9% for anthocyanins after a 5-min extraction (Table 5). After 15 min of extraction, an average of 95.9% total phenols and 100.5% anthocyanins were obtained. Even though extraction yields of both total phenols ($p < 0.001$) and anthocyanins ($p < 0.01$) were significantly increased by the longer solvent contact time, the average increase amounted to less than 2.5%. The relative standard deviations across the different cultivars at 5.9% for total phenols and 4.6% for anthocyanins after a 5-min extraction were almost unaffected by the solvent contact time. Hence, the high extraction efficiencies found after only 5 min of solvent contact were acceptable for a fast extraction protocol. We additionally analyzed the amount extracted in the grape homogenate before solvent extraction and found mean extraction efficiencies of only 35% total phenols and 41% anthocyanins, both with high relative standard deviations across cultivars (17.0% for total phenols and 11.5% for anthocyanins).

In several instances, extraction efficiencies exceeded 100%, which resulted from high turbidity in many of the fast extracts (abs(700) averaged 2.6% and 6.0% of abs(280) and abs(520), respectively), probably caused by insufficient sample clarification. Therefore, extraction efficiencies in Table 5 were to some extent overestimated.

Experiment 3. Final extraction protocol. To avoid potential risk of perturbing the phenolic profile with acidic conditions and because very acidic samples may be incompatible with some analytical methods, a neutralization step was included as a postextraction treatment. The neutralization encompassed addition of sodium hydroxide right after the 5-min extraction. In addition, to overcome the turbidity problems from experiment 2, a filtration step was introduced and a higher centrifugation speed was used prior to dilution with hydrochloric acid. This resulted in acceptable low turbidities, where the abs(700) amounted on average to 0.6% and 1.3% of the abs(280) and abs(520), respectively (data not shown). The 5-min extractions conducted at 40ºC using preheated 50% v/v aqueous ethanol with 0.1 M hydrochloric acid, followed by acid neutralization and sample clarification, gave mean extraction efficiencies of 81.8% total phenols and 91.5% anthocyanins (Table 6). The relative standard deviations of the extraction efficiencies were 6.0% for total phenols and 3.8% for anthocyanins, which shows that the optimized extraction protocol was robust across different grape cultivars.

Stability of phenolics during prolonged solvent contact. To assess the robustness of grape phenolics to contact with acidified solvent, an evaluation of the effect on the phenolic profiles and tannins of extended solvent contact for 5, 10, and 30 min was conducted for a Merlot grape extract (Table 7). From the phenolic profiles obtained, it was not possible to discern any significant changes in the concentrations of the different compounds (Figure 5). An examination of the integrated data indicated that the total concentrations of the main compounds (gallic acid, catechins/flavan-3-ols, hydroxycinnamates, flavonols, and acylated and nonacylated anthocyanins) remained significantly constant during the extended solvent contact (Figure 5, Table 7). The total concentrations of tannins were also constant during the initial 5 min of solvent contact, but decreased by ~9% during the longer solvent contact periods. These results thus clearly demonstrate that the phenols were generally stable during the extraction protocol.

Table 7 Extended exposure of three Merlot grape extracts to the acidified extraction solvent (25% v/v ethanol 0.1 M HCl at 40°C) followed by acid neutralization.

	Mean values		
	Control	**5 min**	**30 min**
Gallic acid (mg/L)	0.80	0.82	0.84
(+)-Catechin (mg/L)	25	27	26
(-)-Epicatechin (mg/L)	18	19	19
Hydroxycinnamate[a]	1.97	2.08	2.06
Flavonol[b]	36	38	39
Anthocyanin[c]	189	188	190
Nonacylated[c]	141	140	141
Acylated[c]	48	48	49
Tannin[d,e]	726[x]	728[x]	662[y]

[a]mg/L caffeic acid equivalents.
[b]mg/L rutin equivalents.
[c]mg/L malvidin-3-glucoside equivalents.
[d]Measured in duplicate and expressed in mg/L catechin equivalents.
[e]No superscript letter indicates that values were not significantly different; different superscript letters indicate significantly different values during extended exposure to the extraction solvent.

Conclusions

In statistically planned experiments, extraction temperature and the concentrations of ethanol and hydrochloric acid were found to significantly affect extraction of total phenols and anthocyanins from grapes. An optimized extraction protocol, giving high average extraction efficiencies of 81.8% total phenols and 91.5% anthocyanins from grapes, was obtained with only 5 min of solvent contact time using 50% v/v aqueous ethanol with 0.1 M HCl at 40°C and a 1:1 w/v grape homogenate:solvent ratio, followed by acid neutralization and sample clarification. The relative standard deviations of the extraction efficiencies across eight grape cultivars were 6.0% for total phenols and 3.8% for anthocyanins, which corroborated the robustness of the protocol across different grape varieties.

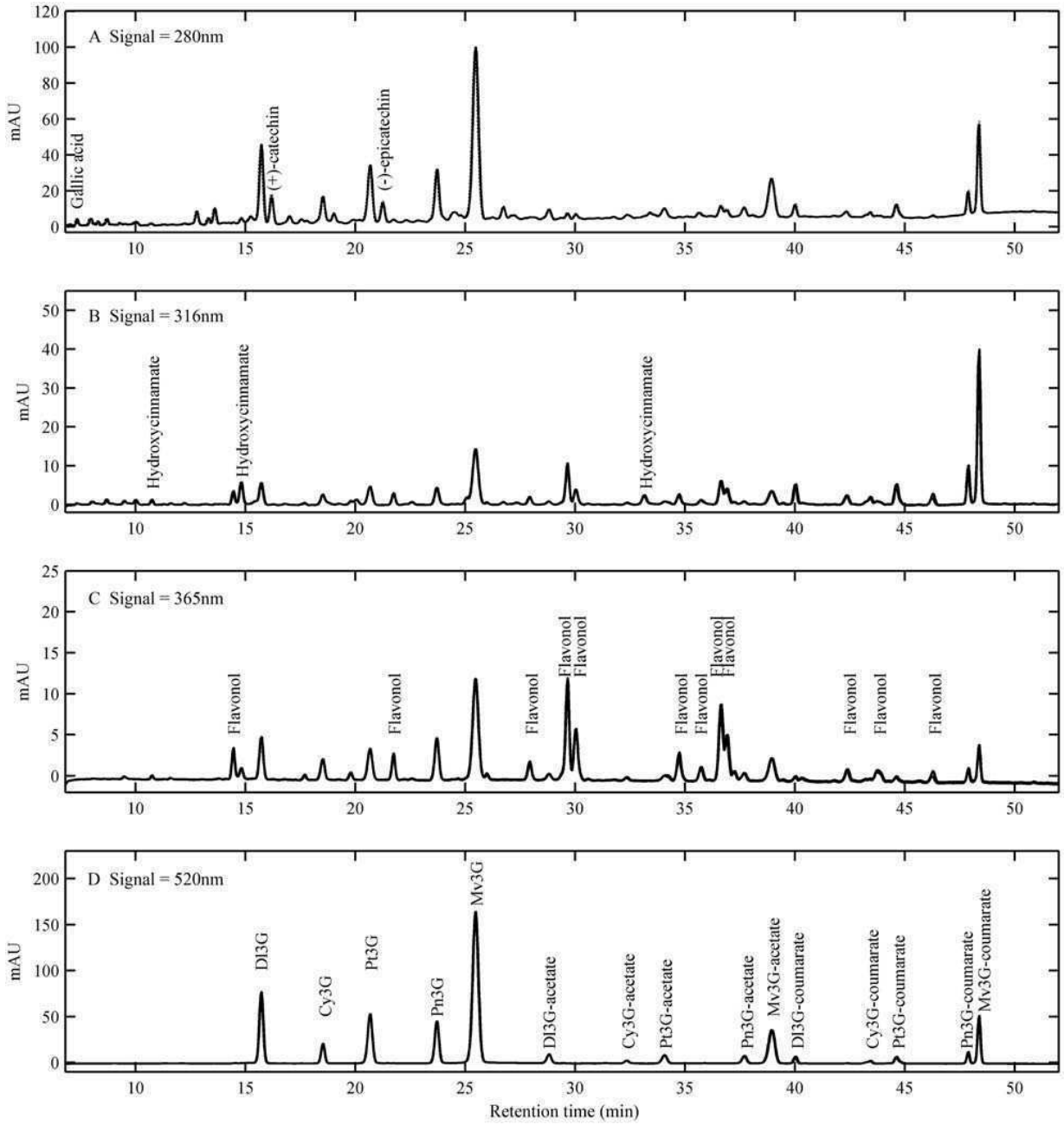

Figure 5 Overlaid HPLC chromatograms of a Merlot grape extract: untreated and exposed to the acidified extraction solvent (25% v/v ethanol, 0.1 M HCl at 40°C) for 5 and 30 min followed by acid neutralization. Chromatograms are shown at (**A**) 280 nm, (**B**) 316 nm, (**C**) 365 nm and (**D**) 520 nm. Dl3G: delphidin-3-glucoside, C3G: cyanidin-3-glucoside, Pt3G: petunidin-3-glucoside, Pn3G: peonidin-3-glucoside, and Mv3G: malvidin-3-glucoside.

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Paper II

Jensen, J.S., Werge, H.H.M., Egebo, M., and Meyer, A.S. 2008. Effect of Wine Dilution on the Reliability of Tannin Analysis by Protein Precipitation. Am. J. Enol. Vitic. 59:103-105.

Technical Brief

Effect of Wine Dilution on the Reliability of Tannin Analysis by Protein Precipitation

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Abstract: A reported analytical method for tannin quantification relies on selective precipitation of tannins with bovine serum albumin. The reliability of tannin analysis by protein precipitation on wines having variable tannin levels was evaluated by measuring the tannin concentration of various dilutions of five commercial red wines. Tannin concentrations of both very diluted and concentrated samples were systematically underestimated, which could be explained by a precipitation threshold and insufficient protein for precipitation, respectively. Based on these findings, we have defined a valid range of the tannin response in the protein precipitation-tannin assay, which suffers minimally from these problems.

Key words: wine, tannin analysis, protein precipitation

Tannins play an important role in the mouthfeel properties and color stability of red wines and are therefore related to wine quality (Kennedy et al. 2006, Singleton and Trousdale 1992). However, reliable quantitative analysis of wine tannins is challenged by the chemical diversity of tannins. Various analytical methods for tannin analysis have been described and reviewed elsewhere (Herderich and Smith 2005, Makkar 1989, Schofield et al. 2001). Tannin analysis by protein precipitation was recently reintroduced as a fast and precise tool for measuring tannins in grapes and wines (Harbertson et al. 2003) and has been recommended for applications within winery settings (Harbertson and Spayd 2006). The method relies on tannins being separated by precipitation with bovine serum albumin (BSA), redissolved, and measured by a color reaction with ferric chloride (Hagerman and Butler 1978, Harbertson et al. 2003).

Tannins determined by protein precipitation have a particularly good correlation with astringency, as compared with some of the other available methods for measuring tannins or related polyphenols (Kennedy et al. 2006). While the mechanism for the precipitation is not fully understood, the existence of threshold levels for tannin precipitation to occur have been reported (Hagerman and Butler 1978, Hagerman and Robbins 1987). The purpose of this study was to investigate the influence of dilution degree on the reliability of tannin analysis by protein precipitation.

Materials and Methods

Chemicals and wines. Five commercial wines with both low and high tannin concentrations were purchased locally: wine 1, Atacama, Cabernet Sauvignon 2005, Chile; wine 2, Cahors Cuvée Prestige, Malbec 2003, France; wine 3, Cecchi–Chianti Classico, Sangiovese 2004, Italy; wine 4, Argento–Mendoza, Malbec 2005, Argentina; and wine 5, Valpolicella Ripasso–Cantina de Soave, Corvina 2004, Italy. Chemicals for tannin analysis were all of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO): bovine serum albumin (BSA, fraction V powder), tartaric acid, sodium dodecyl sulfate (SDS), acetic acid, sodium chloride, triethanolamine, (+)-catechin hydrate, and ferric chloride hexahydrate

Tannin analysis by protein precipitation. All wines were analyzed in duplicates by the precipitation method as described by Harbertson and colleagues (Harbertson et al. 2003) with a few modifications. Prior to analysis the wines were diluted in a model wine solution of 12% v/v ethanol containing 5 g/L tartaric acid, which had been adjusted to pH 3.3 with NaOH. The tannin protein precipitate was formed by mixing 0.5 mL diluted wine and 1 mL BSA solution (containing 1 mg BSA/mL dissolved in a buffer of aqueous 0.2 M acetic acid and 0.17 M sodium chloride adjusted to pH 4.9) for 30 min. Adding the more acidic wines to the BSA solution only slightly decreased the pH, consistently giving final pH values ≥4.8. The precipitate was centrifuged 5 min at 14,000 *g* (Centrifuge 5415D, Eppendorf AG, Hamburg, Germany) to form a pellet and the supernatant was discarded. The pellet was washed twice: each time with 0.25 mL of

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the pH 4.9 buffer, discarding the supernatant after centrifugation for 3 min at 14,000 *g*. The rinsed pellet was redissolved in 1.5 mL buffer (containing 5% w/v SDS and 5% v/v triethanolamine and adjusted to pH 9.4 with HCl) by constant mixing for 20 min. The background (A^{BG}) was measured in a spectrophotometer (Cary 300, Varian, St. Helens, Australia) as the absorbance at 510 nm of 1 mL redissolved solution in a 10-mm semimicro cuvette (Brand, Wertheim, Germany), which was subsequently mixed with 125 µL ferric chloride solution (11.4 mM ferric chloride in 11.4 mM aqueous HCl). The final absorbance at 510 nm (A^{FeCl3}) was measured after 10 min, and the tannin response was calculated as 1.125 times the final absorbance minus the background absorbance. Accounting for dilutions, the tannin concentration was calculated and expressed as mg catechin equivalents (CE) per L from a standard curve of the color reaction between catechin and ferric chloride.

Results and Discussion

The linearity of the tannin response was evaluated by analyzing the tannin concentration of various dilutions of five wines (ranging from undiluted to 10 times dilution) and plotting the tannin response against the inverse dilution factor (Figure 1). Within parts of the dilution ranges, there were good linear relationships ($r > 0.999$) between the inverse dilution factor and the tannin response. However, in some cases at low dilutions (i.e., the concentrated wine samples), the tannin response did not increase proportionally with the inverse dilution factor. This deviation from linearity was likely caused by insufficient protein for the precipitation step. Furthermore, all five wines caused negative y-intercepts, which indicated the existence of a threshold level for the precipitation to occur. The variation in the y-intercepts values did not allow assigning these to a constant value and thereby correcting for this systematic error. At low tannin responses, the y-intercept amounted to a high percentage of the response and hence caused tannin estimations that were too low.

The calculated tannin concentration of both very diluted and concentrated samples were systematically underestimated (Figure 2), probably due to a precipitation threshold and insufficient protein for precipitation, respectively. The maximum tannin concentration was determined for each of the five wines (wine 1, 298 mg CE/L; wine 2, 546 mg CE/L; wine 3, 703 mg CE/L; wine 4, 457 mg CE/L; and wine 5, 391 mg CE/L). These concentrations were used as benchmarks for expressing the calculated tannin values as percentages of maximum concentration. The concentrations, ranging from ~300 to 700 mg CE/L, only covered a part of the known high variation in tannin concentration of wines (Harbertson et al. 2003, Heredia et al. 2006). The relation between the tannin response and the measured tannin concentration in percentage of the maximum demonstrated that at both low and high tannin responses the tannin concentrations were underestimated

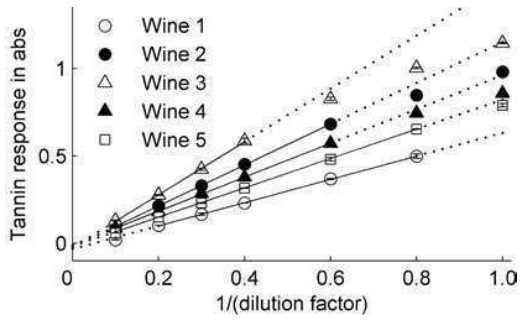

Figure 1 Relationship between the observed tannin response ± SD (in absorbance units) and the inverse dilution factor of dilutions of five wines. Regression lines drawn according to the observed linear range (solid lines) and expanded to the nonlinear range (dotted lines). Regressions for the linear ranges gave $r > 0.999$ for all wines and the following regression lines: wine 1, y = 0.66x-0.032; wine 2, y = 1.17x-0.020; wine 3, y = 1.52x-0.025; wine 4, y = 0.97x-0.011; wine 5, y = 0.84x-0.020.

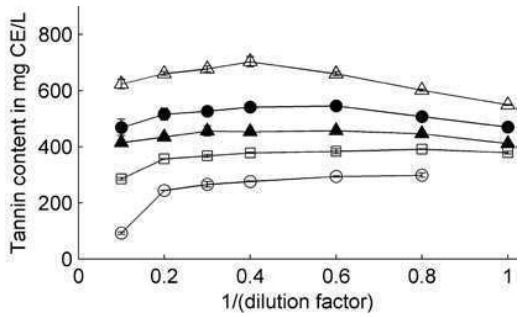

Figure 2 Calculated tannin content ± SD (in mg CE/L) of the five wines (see legend in figure 1) at the different dilutions.

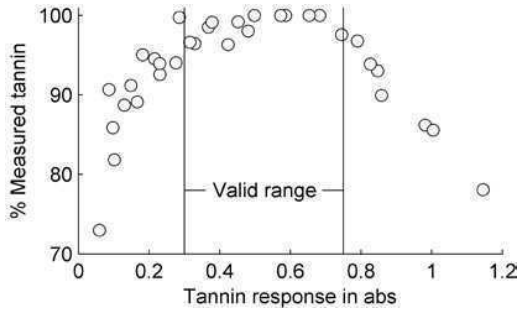

Figure 3 Valid range of tannin response (in absorbance units) defined from the relative proportion of measured tannin concentration to the maximum determined tannin concentration (95 to 100%).

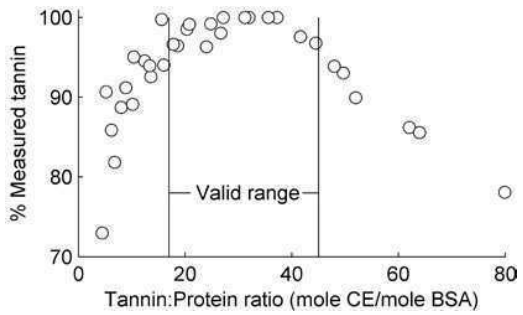

Figure 4 Relation between tannin:protein ratios (in mole CE/mole BSA) and measured tannin concentration (in % of the maximum determined). Lines indicate the range where the measured percentage of tannins are above 95% of the maximum determined tannins.

(Figure 3). Considering the substantial impact of the intercept for highly diluted wines and the need for sufficient protein for the precipitation step, we defined a valid range of the tannin response, where the tannin precipitation suffered minimally from the described problems. By allowing a 5% underestimation, we recommend that the tannin response lies between 0.3 and 0.75 abs under the given conditions. When the original volumes of the protocol are used (Harbertson et al. 2003), the range must be multiplied by a factor of 1.5, which gives a valid range of the tannin response between 0.45 and 1.125 (calculated as $A^{FeCl3} - 0.875 \cdot A^{BG}$). If the tannin response falls outside this range, then there is a risk that the tannin level is underestimated. For example, when wine 3 was measured undiluted, tannin concentration was underestimated by 22% compared with the maximum determined concentration. Likewise, when wine 5 was diluted 10 times, the tannin content was underestimated by 27%. Since these results are obtained using only wine samples, it is advised to check the linearity for other sample types, for example, grape extracts which have much smaller background readings than wines.

Setting a minimum tannin response of 0.3 absorbance units limits the level of tannin that can be reliably quantified to ~140 mg CE/L without prior concentration of the sample. Samples with tannin concentrations less than 140 mg CE/L will most likely be underestimated because of the impact of the precipitation threshold. Even though the saturation stoichiometries of mole tannin per mole BSA are known to vary for different tannins (Hagerman et al. 1998), we recalculated the data to tannin:protein ratios (expressed as mole CE per mole BSA in the precipitation step) and related these to the percent measured tannin levels (Figure 4). From this, the valid range of tannin (in mole CE) to protein (in mole BSA) was between 17 and 45.

Conclusions

The reliability of tannin quantification was hampered by nonlinearity at higher tannin concentrations and the existence of threshold levels for protein precipitation to occur. These two phenomena caused underestimated tannin contrations in samples with either low or high tannin responses. To ensure reliable tannin quantification, we recommend that sample dilutions are carefully carried out to give a tannin response between 0.3 and 0.75 absorbance units under the given conditions.

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Paper III

Jensen, J.S., Demiray, S., Egebo, M., and Meyer, A.S. 2008. Prediction of Wine Color Attributes from the Phenolic Profiles of Red Grapes (*Vitis vinifera*). J. Agric. Food Chem. 56:1105-1115.

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Prediction of Wine Color Attributes from the Phenolic Profiles of Red Grapes (*Vitis vinifera*)

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Knowledge about the relation between grape and wine phenolics is of key interest for the wine industry with respect to being able to predict wine quality from analyses of grapes. Prediction of the phenolic composition and color of experimentally produced red wines from the detailed phenolic composition of the corresponding grapes was investigated using a multivariate approach. Grape extracts and wines were produced from 55 different grape samples, covering 8 different *Vitis vinifera* cultivars: Alicante, Merlot, Syrah, Cinsault, Grenache, Carignan, Cabernet Sauvignon, and Mourvedre. The phenolic composition of the grapes and wines showed that the average ratios between wine and grape phenolics ranged from 0.25 to 7.9 for the different phenolic compounds. Most interestingly, the average ratios were low for anthocyanins (0.31) and tannins (0.32), intermediate for (+)-catechin (0.75) and polymeric pigments (0.98), and high for gallic acid (7.9). Individual wine phenolics in general correlated well with several grape phenolics, indicating that a multivariate approach might be advantageous for prediction of wine phenolics from grape phenolics analysis. However the use of multivariate prediction of individual wine phenolics from the complete grape phenolic composition only improved the prediction of wine polymeric pigments, whereas wine anthocyanins were predicted with the same precision as from the direct relation with grape anthocyanins. Prediction of color attributes of pH normalized experimental wines from the phenolic profiles of grapes was accomplished using a multivariate approach. The correlation between predicted and measured total wine color was high ($r = 0.958$) but was very similar to the correlation coefficient obtained for the direct relation between grape anthocyanins and total wine color ($r = 0.961$). Color due to copigmentation, color due to anthocyanins, and color intensity were also predicted well.

KEYWORDS: Polyphenols; red grapes; red wine; wine color; correlation; prediction.

INTRODUCTION

It has long been recognized that the color intensity of young red wines to some extent correlates positively with the overall wine quality (*1, 2*). It is also known that the color of red wine, to a large degree, depends on its phenolic composition, notably the level of anthocyanins, anthocyanin derivatives, and polymeric pigments (*3–5*). The polyphenols of red wines also impact the taste and mouth-feel properties (*6*). During the red winemaking process the polyphenols are mainly extracted from the grapes during the 5−14 days of maceration, during which the gradually increasing ethanol concentration, resulting from the fermentation, progressively enhances the extraction (*7*). However, even with prolonged maceration, the extraction of polyphenols rarely exceeds 50% of the total grape phenolic content (*8*). In addition, the extraction of polyphenols from grapes is affected by the winemaking conditions, including, in particular, the fermentation temperature, must freezing, skin to juice ratio, maceration time, and enzyme additions (*9*). All of this complicates the establishment of a direct relationship between grape and wine polyphenols. Even though polyphenols undergo several changes and enter into different types of reactions during winemaking — in particular during the fermentation and maturation steps — the main premise of our current research work on understanding quality parameters of red wine is that the polyphenols present in the grapes have a significant influence on the color of the finished wines. In turn, this has led to the hypothesis that it may be possible to predict the wine color from the levels and the profile of the grape polyphenols.

The two most abundant classes of polyphenols found in grapes are anthocyanins and condensed tannins (**Figure 1**). Anthocyanins are almost exclusively located in the outer layers of the grape skin and, under acidic conditions, are highly colored compounds, which are responsible for the color of red grapes (*10*). Tannins are located in the grape seeds and skin and are highly associated with the mouth-feel properties of wine but have also been reported to affect the color development during wine maturation (*3*). Despite these known associations between certain grape polyphenols and wine color attributes, surprisingly

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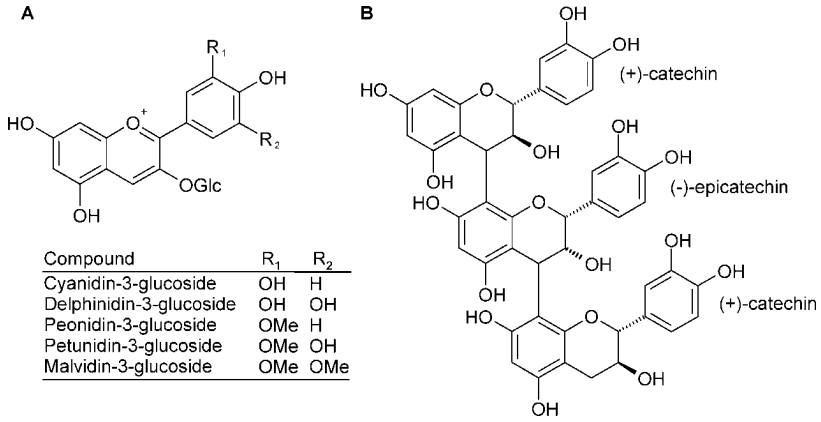

Figure 1. Chemical structures of the unacylated anthocyanins found in *Vitis vinfera* and a hypothetical trimeric procyanidin (tannin) molecule.

Table 1. Grape Sugar Content and Wine Alcohol Levels for the Studied Cultivars[a,b,c]

	grape sugar (°Brix)			wine alcohol (% v/v)		
cultivar	range	mean	SD	range	mean	SD
all samples	18.5–25.6	22.8	1.8	10.4–15.4	13.6	1.2
Alicante	18.6–21.1	19.6 ab	1.1	10.4–12.9	11.5 ab	1.0
Cabernet Sauvignon	21.3–24.9	22.8 bcdef	1.8	12.5–14.4	13.3 bcde	0.9
Carignan	20.4–22.3	21.4 abcd	0.8	12.0–13.2	12.8 abcd	0.5
Cinsault	18.5–24.6	21.8 bcde	2.7	10.5–14.8	13.1 bcd	1.8
Grenache	20.7–23.3	22.5 bcdef	1.2	12.3–14.2	13.6 bcde	0.9
Merlot	21.2–25.6	23.7 def	1.1	12.1–15.4	14.2 de	0.8
Mourvedre	19.8–22.2	21.3 abcd	1.1	11.9–13.3	12.9 bcd	0.7
Syrah	20.9–25.2	23.3 cdef	1.8	11.8–15.0	13.5 bcde	1.4

[a] Four different samples were analyzed for each cultivar, except Merlot with 27 different samples. [b] ANOVA showed significant differences ($p < 0.05$) between cultivars for both grape sugar and wine alcohol levels. [c] Values in the same column followed by the same letter are not significantly different ($p < 0.05$) from a LSD test.

few studies have systematically investigated the overall relation between grape and wine polyphenols. Through the use of an extensive extraction protocol, Iland found a direct linear relation ($R^2 = 0.82$) between grape anthocyanins and wine color density (*11*). González-Neves et al. found that the correlation coefficients between wine color intensity and grape anthocyanins were of similar magnitude irrespective of extracting at pH 1 or at a typical pH of red wine (*12*). The data reported by Romero-Cascales et al. also indicated that anthocyanins extracted at a typical pH of red wine correlated to wine color (*13*). However their results, obtained using five grape samples, also indicated that the extractability of anthocyanins from grapes affected the significance of the correlation (*13*).

Because wine color not only relates to the levels of anthocyanins but also to the level of other phenolic compounds (*3, 5, 14*), the use of a multivariate approach on several grape phenolic parameters could lead to a better understanding of the relation between grape phenolics and wine color. The objective of this study was to investigate the relationship between the polyphenols in grapes and those in corresponding young wines and their color attributes at the end of the alcoholic fermentation. We here report the identification of such a relationship and thus demonstrate that at least some wine color attributes can be predicted from the phenolic composition of grapes.

MATERIALS AND METHODS

Chemicals. Technical grade 96% v/v ethanol (V&S Distillers, Aalborg, Denmark) and analytical grade hydrochloric acid (Merck, Darmstadt, Germany) were used for preparing solvents for grape extractions. Acetonitrile, *o*-phosphoric acid, gallic acid, (+)-catechin hydrate, (−)-epicatechin, rutin hydrate, and caffeic acid were all of high-performance liquid chromatography (HPLC) grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade malvidin-3-glucoside hydrochloride was purchased from Extrasynthese (Genay, France). Chemicals for color analysis and protein precipitation: Bovine serum albumin (BSA, fraction V powder), tartaric acid, potassium tartrate, sodium dodecyl sulfate (SDS), triethanolamine (TEA), ferric chloride hexahydrate, potassium disulfite, and acetaldehyde were all of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Instrumentation. HPLC analysis was carried out on an 1100 series HPLC instrument (Agilent, Santa Clara, CA, USA) equipped with a vacuum degasser, a quaternary pump, an autosampler, a thermostatted column compartment, and a diode array detector. Ultraviolet-visible (UV/vis) absorbance readings were measured on a Lambda2 spectrophotometer (PerkinElmer, Waltham, MA, USA). Infrared spectra in the mid-infrared range (926−5012 cm^{-1}) were measured by Fourier transform interferometry on a Winescan FT120 spectrometer (FOSS, Hillerød, Denmark) equipped with a liquid flow system and a 37 μm calcium fluoride cuvette, thermostatted at 40 °C.

Grape Material. Fifty-five different grape samples covering eight different red cultivars (Alicante, Merlot, Syrah, Cinsault, Grenache, Carignan, Cabernet Sauvignon, and Mourvedre) of *Vitis vinifera* were collected from different fields in the south of France in August and September, 2005 and 2006. For each sample, mature grapes were manually picked and stored immediately at −30 °C.

Each sample was manually destemmed and mixed well while frozen. Sample aliquots of 100−250 g were taken from the same lot of frozen grapes for determination of grape sugar (100 g), grape extractions (150 g), and for microscale wine making (250 g). For sugar determination, the grapes were thawed and manually squeezed to obtain a juice. The grape juice was centrifuged (15 000*g*, 10 min) and filtered through a Whatman grade 4 cellulose filter, and the infrared spectra were recorded on the Winescan. The sugar levels (see **Table 1**) of the grapes were then determined from the infrared spectra via a calibration model for grape juice (FOSS, Hillerød, Denmark).

Grape Extraction Procedure. The grapes were extracted using a fast extraction protocol, found to extract a high proportion of the grape polyphenols (*15*). Briefly, the grapes were thawed and homogenized

thoroughly with an Ultra-Turrax T25 high-speed homogenizer (IKA-Werke & Co. GmbH KG, Janke & Kunkel, Staufen, Germany). Extraction was conducted by mixing a 1:1 (w/v) ratio of grape homogenate and acidic (0.1 M HCl) aqueous ethanol (50% v/v) at 40 °C, followed by neutralization of the added hydrochloric acid with a stoichiometric amount of sodium hydroxide (5M). The sample was centrifuged (15 000*g*, 10 min), filtered through a Whatman grade 4 cellulose filter, and filtrates were frozen for later analyses (HPLC and protein precipitation assay). Total phenols and anthocyanins of the unfrozen filtrates were measured as outlined below. The phenolic contents per grape mass unit were calculated from the diluted extracts using an experimentally determined average volume of extracted sample (V_s) at 1.891 mL of extract per g of grape.

Measurement of Total Phenols and Anthocyanins by Spectroscopy. Samples were centrifuged for 5 min at 23 000*g*, diluted in 1 M HCl, and after one hour the absorbances at 280 and 520 nm were measured in 10 mm quartz cuvettes. The anthocyanin content (abbreviated Anth-spec) was expressed in mg of malvidin-3-glucoside equivalents (ME) per kg of grape from the absorbance at 520 nm (*16, 17*) via use of an extinction coefficient $\epsilon = 58.3$ mL/(mg · cm), found from a standard curve of malvidin-3-glucoside, using equation 1. The content of total phenols per kg of grape was calculated and expressed as 0.01 absorbance units at 280 nm (*16, 17*), from equation 2.

$$\text{Anthocyanins (mg/kg)} = 1000V_s\text{DF}\cdot\text{abs}(520\text{ nm})\cdot 1/\epsilon \quad (1)$$

$$\text{Total phenols (0.01abs)} = 1000V_s\text{DF}\cdot\text{abs}(280\text{ nm})/100 \quad (2)$$

where DF is the dilution factor of the extract in 1 M HCl, V_s is the volume of extracted sample per g of grape, and 1 is the cuvette path length in cm.

Wine Making Procedure. Wines were produced in microscale by the following protocol: approximately 250 g of grapes were weighed and thawed overnight at 5 °C, supplemented with 69 mg/L potassium disulfite (corresponding to 40 mg/L SO_2) and gently crushed for 1 min in a Stomacher laboratory-blender (Seward, Thetford, UK), without crushing the grape seeds. The crushed grapes were transferred to a 500 mL glass bottle, sealed with an airlock, and heated to 25 °C in a water bath. The crushed grapes were supplemented with diammonium hydrogenphosphate (100 mg/L) and inoculated with approximately 0.2 g/L *Saccharomyces cerevisiae* dry yeast (Vinoflora Ruby.ferm, Chr. Hansen, Hoersholm, Denmark), from a yeast starter culture prepared the previous day and kept at 25 °C. The wines were fermented in the dark for a total of 14 days in a thermostatted water bath at 25 °C. Two days after inoculation, the headspace of each fermenting wine sample was carefully replaced with air, and the bottle was shaken to ensure sufficient oxygen for the yeast. During the entire period, the cap was broken twice a day by manually shaking the bottles. The conversion of sugars to ethanol was monitored during the fermentation for a few selected fermentations and was determined for all wines after 14 days of fermentation by measuring the infrared spectra on the Winescan and predicting the level of ethanol and sum of glucose and fructose via a calibration model for fermenting must samples (FOSS, Hillerød, Denmark). After 14 days of fermentation the wines were all fermented to dryness (less than 4 g/L of glucose + fructose, except one Syrah wine sample having 11 g/L glucose + fructose) and had alcohol levels ranging from 10.4 to 15.4% v/v (**Table 1**). The wines were weighed and separated from the pomace by centrifugation (15 000*g*, 10 min) and filtered through a Whatman grade 4 cellulose filter. The wines were then flushed with nitrogen and allowed to settle at 8 °C for one week in airtight flasks, and wine color attributes were measured as described below. In addition, total phenols and anthocyanins of the wines were estimated by spectroscopy (eqs 1 and 2). Samples were frozen for later phenolic analyses (HPLC and protein precipitation). An average yield of sample volume (V_s) at 0.861 mL of wine per g of grape was found and used to report phenolic content based on the original grape mass.

Analysis of Phenolic Compounds by HPLC. Phenolic compounds of both extracts and wines were determined by HPLC using a newly developed method (*15*). Briefly, the separation of the phenolics was conducted on a Gemini C18 column (150 mm × 4.6 mm, 3 μm particle size, 110 Å pore size) from Phenomonex (Phenomenex, Torrance, CA, USA) with a 4 × 3 mm guard column of the same material used as stationary phase at 40 °C. The solvents were: solvent A (water with 0.20 M *o*-phosphoric acid and 3% v/v acetonitrile, adjusted to pH 1.5 with aqueous sodium hydroxide) and solvent B (a 1:1 v/v mixture of solvent A and acetonitrile). A constant flow of 0.5 mL/min was applied with a linear gradient elution profile of: 0 min (11% solvent B), 40 min (40% solvent B), 50 min (60% solvent B), 53 min (100% solvent B), 60 min (100% solvent B), 61 min (11% solvent B), and 66 min (11% solvent B). Prior to injection, each sample was centrifuged at 23 000*g* for 5 min, filtered through a Phenex 0.45 μm nylon syringe filter (Phenomenex, Torrance, CA, USA), and stored under nitrogen until analysis. The injection volume was 10 μL. The compounds were identified according to their retention times and spectral properties. Gallic acid, (+)-catechin, and (–)-epicatechin were quantified at 280 nm from external standard curves of authentic standards. On the basis of spectral identification and external standard curves, hydroxycinnamates (abbreviated hydroxycin) were quantified at 316 nm as caffeic acid equivalents (CFAE), flavonols were quantified as rutin equivalents (RUE) at 365 nm, and anthocyanins (abbreviated Anth-HPLC) were quantified as malvidin-3-glucoside equivalents (ME) at 520 nm (*15, 18*).

Protein Precipitation Assay. Monomeric pigments (MP), polymeric pigments (PP), small polymeric pigments (SPP), large polymeric pigments (LPP), and tannins were measured using a slightly modified method of Harbertson et al. (*19*). Briefly, the method relies on that tannins are precipitated with bovine serum albumin, redissolved, and measured by a color reaction with ferric chloride. The polymeric pigments are measured by bleaching with sulfite and SPP and are defined as the fraction of the polymeric pigments that is not precipitated with bovine serum albumin. Prior to analysis, wine or grape extracts were filtered through Phenex 0.45 μm nylon syringe filters and diluted in a model wine solution of 12% v/v ethanol containing 5 g/L of tartaric acid, which had been adjusted to a pH value of 3.3 with NaOH. The modifications to the original method were as follows. The precipitation step was conducted for 30 min instead of 15 min, the centrifugation speed for forming the tannin−protein pellet was increased from 13 500*g* to 14 000*g*, and finally, the SDS/TEA buffer volume for redissolving the tannin−protein pellet was increased from 0.875 to 1.5 mL to allow background measurement (A^{BG}) on a 1 mL sample, which was then reacted with 0.125 mL of iron chloride (11.4 mM $FeCl_3$ in 11.4 mM aqueous HCl), and the absorbance measured after 10 min (A^{FeCl_3}). Dilution of the samples in the model wine solutions was carried out to give a tannin response (calculated as $1.125A^{FeCl_3} - A^{BG}$) between 0.3 and 0.75, which was defined as the valid range of the assay. Accounting for the dilutions MP, PP, SPP, and LPP were expressed as absorbance units, and tannins were expressed as mg catechin equivalents (CE)/mL from a standard curve of the color reaction between catechin and ferric chloride.

Wine Color Measurements. Prior to all color measurements, wines were normalized to pH 3.6, by adjusting with a minimum volume of aqueous NaOH or HCl and filtered through a Phenex 0.45 μm nylon syringe filter (Phenomenex, Torrance, CA, USA). Boulton's color assay was used to determine the total wine color and wine color due to copigmentation, anthocyanins, and polymeric pigments, respectively (*20*). Full UV/vis transmission spectra (250–750 nm) of the pH adjusted and filtered wines were measured in 1 mm quartz cuvettes. The absorbance values at 420 and 520 nm were used to calculate the color intensity and tonality (*21*).

Repeatability. To asses the experimental error, triplicate grape extractions and wines were produced using the described protocols and were analyzed for three different samples (Cinsault, Merlot, and Alicante). The repeatability (Rep) for each measured variable was calculated as the average standard deviation, obtained from the pooled average variance of the three samples (*22*), divided by the average value (eq 3).

$$\text{Repeatability (in \%)} = \frac{100}{\text{average}(y)}\sqrt{\frac{1}{n(J-1)}\sum_{i=1}^{n}\sum_{j=1}^{J}(y_{ij} - \text{average}(y_i))^2} \quad (3)$$

where n is the number of samples, J is the number of replicate measurements, i is the sample number, j is the replicate measurement number, and y is the value of the measured variable.

Table 2. Mean Values of the Phenolic Composition of Grape Extracts (per kg of Grape) for the Studied Cultivars[a,b,c]

phenolic compound	all samples[d]	Alicante	Cabernet Sauvignon	Carignan	Cinsault	Grenache	Merlot	Mourvedre	Syrah
total phenols (0.01 abs)	1518 (±23%)	2064 c	1585 b	1210 a	876 a	1183 a	1585 b	1621 b	1638 b
anth-spec (mg ME/kg)	1258 (±43%)	2622 f	1381 cde	1265 cde	608 ab	800 abc	1142 bcd	1398 cde	1514 de
MP (abs)	3.4 (±51%)	8.3 f	3.6 cde	3.4 cde	1.7 ab	2.0 abc	2.9 bcd	3.8 cde	4.3 de
SPP (abs)	0.45 (±37%)	0.87 e	0.54 cd	0.33 ab	0.30 ab	0.36 abc	0.41 abc	0.49 bcd	0.53 cd
LPP (abs)	0.53 (±50%)	0.63 bcd	0.65 bcd	0.32 abc	0.23 ab	0.32 abc	0.56 bcd	0.68 cd	0.69 cd
tannins (mg CE/kg)	2662 (±28%)	1826 abc	3492 fg	1923 abc	1303 ab	2204 bcd	2934 def	3347 efg	2701 cde
PP (abs)	0.98 (±38%)	1.5 de	1.2 cde	0.66 abc	0.53 ab	0.68 abc	0.97 bcd	1.2 cde	1.2 cde
gallic acid (mg/kg)	3.4 (±52%)	2.2 abcd	3.3 bcd	1.1 ab	2.5 abcd	1.6 ab	4.7 de	1.1 ab	3.6 bcde
(+)-catechin (mg/kg)	127 (±47%)	101 cd	159 e	40 ab	46 ab	104 cd	170 e	54 abc	93 bcd
(−)-epicatechin (mg/kg)	114 (±51%)	129 cde	97 bcd	23 ab	61 abc	60 abc	157 de	32 ab	105 bcd
hydroxycin. (mg CFAE/kg)	54 (±57%)	122 b	40 a	41 a	38 a	98 b	48 a	37 a	43 a
flavonols (mg RUE/kg)	254 (±34%)	350 cd	295 bcd	244 bc	105 a	230 bc	247 bc	297 bcd	307 bcd
anth-HPLC (mg ME/kg)	1267 (±42%)	2607 e	1339 bcd	1371 bcd	576 a	778 a	1160 bc	1389 bcd	1521 cd

[a] Four different samples were analyzed for each cultivar, except Merlot with 27 different samples. [b] ANOVA showed significant differences ($p < 0.05$) between cultivars for all 13 phenolic compounds. [c] Values in the same row followed by the same letter are not significantly different ($p < 0.05$) from a LSD test. [d] The mean value (±relative SD) for all 55 samples.

Table 3. Mean Values of the Phenolic Composition of Wines (per kg of Grapes Used for Winemaking) for the Studied Cultivars[a,b,c]

phenolic compound	all samples[d]	Alicante	Cabernet Sauvignon	Carignan	Cinsault	Grenache	Merlot	Mourvedre	Syrah
total phenols (0.01 abs)	665 (±27%)	887 f	636 bcd	491 abc	347 ab	391 ab	754 de	596 bcd	711 cde
anth-spec (mg ME/kg)	518 (±38%)	944 e	568 bcd	515 bc	239 a	278 a	497 bc	524 bcd	692 cd
MP (abs)	2.2 (±46%)	4.7 f	2.1 bcde	2.0 bcd	0.96 a	1.1 a	2.2 bcd	2.1 bcde	2.9 ce
SPP (abs)	0.67 (±38%)	1.1 g	0.79 cdef	0.48 abd	0.31 ab	0.32 ab	0.68 cde	0.69 bcdef	0.86 def
LPP (abs)	0.28 (±50%)	0.55 d	0.27 bc	0.21 abc	0.10 ab	0.12 ab	0.30 bc	0.24 abc	0.31 bc
tannins (mg CE/kg)	860 (±38%)	681 abcde	807 cde	547 abcd	422 abc	426 abc	1121 f	675 abcde	702 bcde
PP (abs)	0.95 (±40%)	1.7 e	1.1 cd	0.70 abc	0.41 ab	0.44 ab	0.98 cd	0.93 bcd	1.2 cd
gallic acid (mg/kg)	23 (±42%)	19 abcde	25 cdef	9.5 abc	13 abcd	23 bcdef	29 def	14 abcd	22 bcdef
(+)-catechin (mg/kg)	94 (±49%)	66 cde	107 f	29 abc	32 abcd	63 cde	132 g	48 abcde	59 bcde
(−)-epicatechin (mg/kg)	77 (±57%)	59 bcd	60 bcd	12 ab	37 abc	31 abc	114 e	24 ab	71 cd
hydroxycin. (mg CFAE/kg)	12 (±79%)	35 d	5.4 ab	11 abc	11 abc	17 bc	10 abc	6.1 ab	9.7 abc
flavonols (mg RUE/kg)	86 (±45%)	94 bc	75 abc	90 bc	37 ab	39 ab	94 bc	99 bc	108 bc
anth-HPLC (mg ME/kg)	392 (±39%)	725 e	411 bcd	409 bcd	187 a	233 a	378 bc	384 bcd	491 cd

[a] Four different samples were analyzed for each cultivar, except Merlot with 27 different samples. [b] ANOVA showed significant differences ($p < 0.05$) between cultivars for all 13 phenolic compounds. [c] Values in the same row followed by the same letter are not significantly different ($p < 0.05$) from a LSD test. [d] The mean value (±relative SD) for all 55 samples.

Statistical and Multivariate Data Analysis. Analysis of variance (ANOVA) and the least significant differences (LSD) test (*23*) was carried out to detect differences in the phenolic contents between the grape cultivars and to categorize the significant differences ($p < 0.05$), using MATLAB R14 (MathWorks, Natick, MA, USA) and the Statistics Toolbox 5.0.2 (MathWorks, Natick, MA, USA). Multivariate data analysis was carried out in MATLAB using the PLS toolbox 4.02 (Eigenvector Research, Natick, MA, USA). Principal component analysis (PCA) was performed to visualize the main variations between samples, groupings of samples, and the relation between samples and the phenolic composition. Calibration models were developed with partial least-squares (PLS) regression using leave-one-out cross validation. The optimal number of factors in the model (termed latent variables) was determined by minimizing the root-mean-square error of cross validation (RMSECV). Other model statistics included the correlation coefficient (r) between the actual and predicted values, the root-mean-square error of calibration (RMSEC), and the residual predictive deviation (RPD), defined as the standard deviation of the sample population divided by the standard error in cross validation (SECV).

RESULTS AND DISCUSSION

Phenolics in Grapes and Wines. The determination of the phenolic composition of grapes is strongly dependent upon the employed extraction method. Most reported extraction methods require long extraction times, use of different organic solvents, or multistep sample preparation (*16, 24, 25*). In this study, we have used a newly developed extraction method, by which a high degree of extraction from the grapes has been obtained using acidified aqueous ethanol and short solvent contact time (*15*).

To allow direct comparisons of the phenolic levels in grapes and wines, the phenolic levels were reported in per kg of grape used for grape extraction and winemaking, respectively. The average level of tannins amounted to 2662 mg CE/kg of grape in the grapes (**Table 2**) and 860 mg CE/kg of grape in the wines (**Table 3**). On average, anthocyanins determined by HPLC amounted to 1267 mg ME/kg of grape in the grapes (**Table 2**) and 392 mg ME/kg of grape in the wines (**Table 3**). Considerable amounts of flavonols, (−)-epicatechin, (+)-catechin, hydroxycinnamates, and gallic acid (mostly in wines) were also detected in both grapes and wines (**Tables 2** and **3**). The average levels of small polymeric pigments (0.67 abs) and large polymeric pigments (0.28 abs) in wines (**Tables 2** and **3**) were at least two times lower than the levels reported in commercial wines (*19*). These relatively low levels may be a result of these polymeric pigments being primarily formed during the maturation process (*5*), which was not included in this study.

For both grapes and wines the differences between the eight cultivars were rather complex, and typically, the phenolic levels overlapped between several cultivars. Interestingly, the tannin levels of Merlot wines were found to be significantly higher than those in the wines produced from other cultivars (**Table 3**), whereas tannins in Merlot grapes were only significantly higher than the levels found in Cinsault, Carignan, Alicante, and Grenache, but significantly lower than the tannin levels in

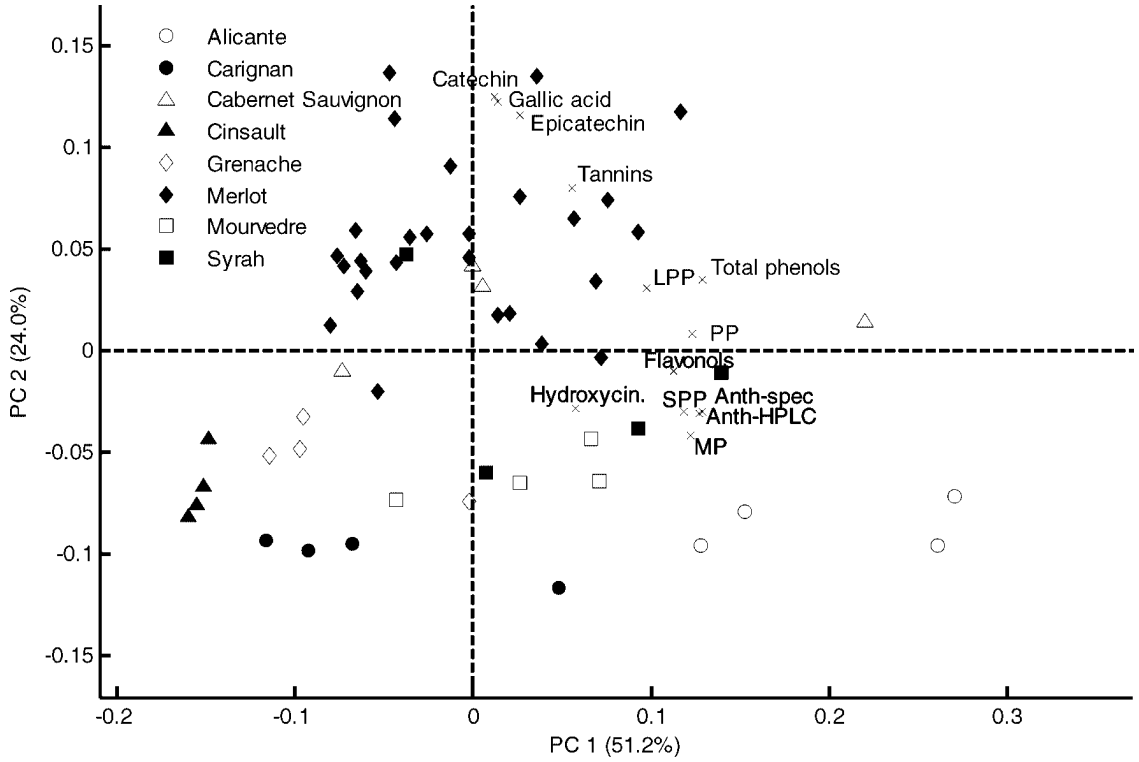

Figure 2. Biplot of scores and loadings from the PCA of the phenolic composition of the grapes.

Mourvedre and Cabernet Sauvignon grapes (**Table 2**). This difference in groupings from grape to wine may be a result of both chemical and physiological differences between the cultivars. Anthocyanin levels in the wines, as determined by HPLC, were consistently the highest in Alicante wines, lowest in Cinsault and Grenache wines and almost similar between the other cultivars (**Table 3**). The same grouping pattern for anthocyanins was found in the grape extracts (**Table 2**), which indicated some similarities between the anthocyanin levels in grapes and wines. The levels of total phenols in grapes seemed to categorize the grapes in three significantly different groups with Alicante having the highest levels; Cabernet Sauvignon, Merlot, Mourvedre, and Syrah having intermediate levels; and Carignan, Cinsault, and Grenache having the lowest levels (**Table 2**). The pattern for total phenols was slightly altered in the wines, in which a bigger overlap between cultivars caused less sharp groupings of the cultivars, which, as for grapes, signified Alicante wines to have high levels; Cabernet Sauvignon, Merlot, Mourvedre, and Syrah wines to have intermediate total phenols levels; and the wines made from Carignan, Cinsault, and Grenache to have low levels of total phenols (**Table 3**).

Sample Characterization by Principal Component Analysis of Phenolics. Principal component analysis (PCA) of the phenolic compositions was used to identify the most important differences between the samples and to relate this to both the phenolic compositions and the cultivar. For grape extracts, the first principal component explained 51% of the variation and was associated with anthocyanins, polymeric pigments, flavonols, and hydroxycinnamates (**Figure 2**). The second principal component explained another 24% of the variation and was associated with tannins, catechins, and gallic acid. Some cultivar differences were observed from the two first principal components (**Figure 2**). Merlot grape samples were found to have quite high levels of tannins, catechins, and gallic acid and intermediate levels of anthocyanins and other pigments. Alicante samples had very high levels of anthocyanins and pigments but had intermediate levels of tannins and catechins. Grenache, Cinsault, and Carignan samples were characterized by low levels of all the phenolics. Mourvedre, Syrah, and Cabernet Sauvignon grapes were generally characterized by intermediate levels of phenolics, although considerable sample differences were recorded for Cabernet Sauvignon and Syrah, in particular. In addition, the first two principal components did not capture the actual high tannin levels of Mourvedre extracts (**Figure 2**), probably due to this cultivar simultaneously having low levels of catechins and gallic acid (**Table 2**). PCA on the phenolic composition of wines (**Figure 3**) gave a slightly higher explained variation (57 and 24%), but gave in general similar groupings as found in the PCA of the phenolic composition of grapes. The largest difference between the two PCA plots were that the position of LPPs moved from the first to fourth quadrant from grape to wine, indicating that the relations between LPP levels and the cultivars were slightly different from grapes to wine.

Ratios between Grape and Wine Phenolics. The magnitude of the ratio between the phenolic contents of wines to grapes (**Table 4**) described how large a proportion of the grape phenolics that was recovered in the wine. The average ratio of 0.44 for total phenols was in good accordance with the general observation that extraction of phenols during wine making rarely exceeds 50% (*8*). However, large differences in the wine/grape ratios were observed among the different phenolic compounds. The most striking observation was that the levels of gallic acid were found to be much higher in wines than in grapes, with an average ratio of 7.9 (**Table 4**). Elevated levels in wines versus the corresponding grapes have also been found by others and are suggested to be caused by a release of gallic acid by hydrolysis of gallate esters during wine manufacturing (*26*). Interestingly, there was a difference between the ratios for small and large polymeric pigments (on average 1.5 and 0.57, respectively), which could reflect differences in formation and/or extraction kinetics. Average ratios for (+)-catechin and (−)-epicatechin were 0.75 and 0.66, respectively, which indicated

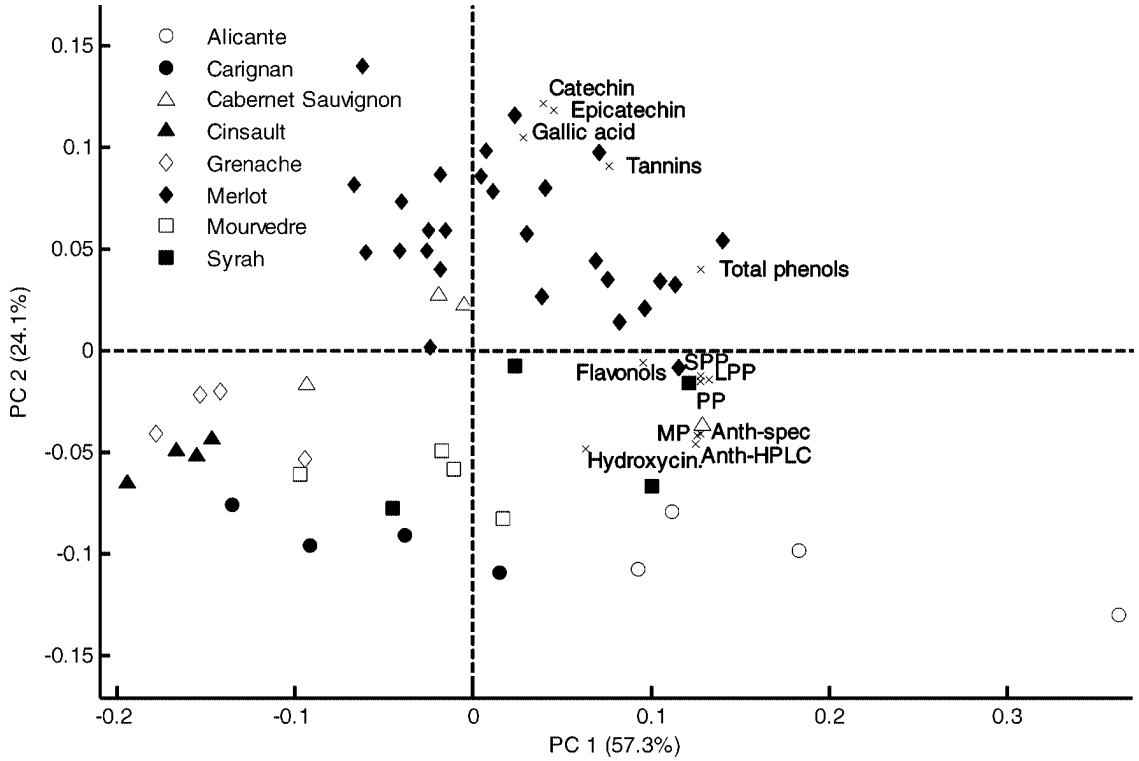

Figure 3. Biplot of scores and loadings from the PCA of the phenolic composition of the wines.

Table 4. Ratio between Phenolic Levels in Grapes and Wines for the Studied Cultivars[a,b,c]

	all samples[d]	Alicante	Cabernet Sauvignon	Carignan	Cinsault	Grenache	Merlot	Mourvedre	Syrah
total phenols	0.44 (±13%)	0.43 cd	0.41 bcd	0.41 bcd	0.40 bcd	0.33 ab	0.48 e	0.37 abc	0.43 cd
anth-spec	0.42 (±12%)	0.36 abcd	0.42 bcdef	0.41 bcdef	0.39 abcde	0.35 abc	0.44 cdef	0.38 abcd	0.47 def
MP	0.66 (±17%)	0.56 ab	0.60 abc	0.61 abc	0.56 ab	0.54 ab	0.74 cd	0.56 ab	0.71 bcd
SPP	1.5 (±24%)	1.3 bcd	1.5 cde	1.5 cde	1.0 abc	0.89 ab	1.7 de	1.4 cde	1.7 de
LPP	0.57 (±43%)	0.86 cde	0.42 abcd	0.66 abcde	0.46 abcd	0.39 abd	0.63 bcde	0.39 abd	0.45 abcd
tannins	0.32 (±27%)	0.37 def	0.22 abc	0.28 bcd	0.33 cde	0.19 ab	0.38 ef	0.20 ab	0.25 abc
PP	0.98 (±24%)	1.1 defgh	0.93 abcdefgh	1.1 cdefgh	0.79 abcde	0.64 abcd	1.1 defgh	0.81 abcdeg	0.97 bcdefgh
gallic acid	7.9 (±47%)	8.7 a	7.6 a	8.8 a	6.0 a	14 b	6.5 a	14 b	5.9 a
(+)-catechin	0.75 (±16%)	0.66 ab	0.67 ab	0.74 abc	0.72 abc	0.60 ab	0.79 bcd	0.90 cd	0.69 abc
(−)-epicatechin	0.66 (±22%)	0.45 abc	0.61 abcdf	0.51 abcd	0.64 bcdef	0.51 abcd	0.74 def	0.74 cdef	0.65 bcdef
hydroxycin.	0.25 (±79%)	0.27 abcde	0.15 abc	0.42 bcde	0.48 cde	0.17 abcd	0.23 abcd	0.18 abcd	0.24 abcde
flavonols	0.34 (±28%)	0.27 abcde	0.24 abcd	0.36 cdef	0.35 cdef	0.17 abc	0.37 def	0.31 bcdef	0.38 def
anth-HPLC	0.31 (±12%)	0.28 abd	0.31 abcd	0.30 abcd	0.33 abcd	0.30 abcd	0.33 bcd	0.28 abd	0.33 abcd

[a] Four different samples were analyzed for each cultivar, except Merlot with 27 different samples. [b] ANOVA showed significant differences ($p < 0.05$) between cultivars for the ratios of all phenolic compounds, except hydroxycinnamates ($p = 0.161$) and anthocyanins HPLC ($p = 0.131$). [c] Values in the same row followed by the same letter are not significantly different ($p < 0.05$) from a LSD test. [d] The mean value (± relative SD) of the ratios for all 55 samples.

that the majority of these compounds were recovered in the wines (**Table 4**). An average ratio of 0.32 for tannins showed that tannins were only partly recovered in the wines, which is in accordance with the known slow extraction of tannins from grapes during winemaking (*9*). Ratios for the tannins showed some differences between the cultivars, with notable high ratios for Merlot and Alicante and low ratios for Grenache, Mourvedre, and Cabernet Sauvignon (**Table 4**). On the other hand, for anthocyanins determined by HPLC, the low average ratio of 0.31 was likely caused by the mixed effect of incomplete extraction and chemical transformations of the anthocyanins during wine making. It is well-known that anthocyanins are simultaneously extracted and transformed during the fermentation (*3*). From the ANOVA we were not able to significantly detect cultivar differences in the ratios for anthocyanins ($p = 0.161$) and pairwise LSD tests showed large overlapping of the anthocyanin levels between the cultivars (**Table 4**). Also, considering the relatively small sample variation in the ratios for anthocyanins (CV = 12%, **Table 4**), it seemed that anthocyanins were recovered to a similar extent in the different cultivars during wine making. The average ratio for MP was more than twice as high as anthocyanins determined by HPLC, which showed that MP was not an accurate measure of anthocyanins (**Table 4**). For grapes, the anthocyanin levels determined by spectroscopy (anth-spec) were in good accordance with levels measured by HPLC (**Table 2**) but not for wines (**Table 3**).

Relation between Grape and Wine Phenolics. To investigate how the phenolic composition of grapes and wines correlated, the correlation coefficients between grapes and wines for individual phenolic groups were calculated (**Table 5**). In general, the level of each wine phenolic was best correlated with the level of the same corresponding phenolic compound in grape, with only a few exceptions. Wine tannins were not very well related to any of the phenolics in grapes (**Table 5**), and it was noticed that the best correlations were found with grape tannins ($r = 0.68$), (+)-catechin ($r = 0.67$), (−)-epicatechin ($r = 0.62$), and gallic acid ($r = 0.61$) (**Table 5**). In

Table 5. Correlation Coefficients (r) between the Content of Phenolics in Grape Extracts and Wines for All Samples ($N = 55$)[a]

	wine content												
grape content	total phenols	anth-spec	MP	SPP	LPP	tannins	PP	gallic acid	(+)-catechin	(−)-epicatechin	hydroxycin.	flavonols	anth-HPLC
total phenols	**0.89**	0.85	0.84	0.87	0.80	0.53	0.88	0.30	0.29	0.28	0.35	0.59	0.83
anth-spec	0.68	**0.95**	0.94	0.88	0.81	0.14	0.88	−0.04	−0.12	−0.09	0.51	0.47	0.94
MP	0.61	0.92	**0.92**	0.84	0.77	0.04	0.84	−0.10	−0.19	−0.13	0.53	0.41	0.91
SPP	0.58	0.83	0.82	**0.82**	0.70	0.08	0.80	−0.04	−0.12	−0.07	0.40	0.35	0.81
LPP	0.61	0.57	0.52	0.62	**0.48**	0.38	0.59	0.16	0.16	0.10	0.05	0.54	0.54
tannins	0.54	0.26	0.18	0.37	0.32	**0.68**	0.37	0.41	0.48	0.33	−0.31	0.40	0.21
PP	0.70	0.79	0.74	0.81	0.66	0.31	**0.78**	0.09	0.06	0.04	0.21	0.54	0.75
gallic acid	0.51	0.07	0.08	0.26	0.20	0.61	0.25	**0.64**	0.80	0.90	−0.10	0.15	0.03
(+)-catechin	0.49	0.00	0.04	0.17	0.20	0.67	0.19	0.66	**0.96**	0.85	0.00	0.09	−0.01
(−)-epicatechin	0.60	0.16	0.22	0.35	0.34	0.62	0.36	0.69	0.84	**0.95**	0.12	0.09	0.14
hydroxycin.	0.22	0.36	0.43	0.28	0.35	−0.15	0.32	0.06	−0.08	−0.10	**0.81**	−0.03	0.39
flavonols	0.66	0.81	0.75	0.74	0.71	0.32	0.75	0.01	0.02	−0.09	0.29	**0.78**	0.82
anth-HPLC	0.68	0.95	0.94	0.87	0.80	0.13	0.87	−0.06	−0.12	−0.08	0.51	0.49	**0.94**

[a] Values in bold indicate the correlation coefficients between grape and wine for the same phenolic compounds.

Table 6. Direct and Multivariate Relation between Grape and Wine Phenolics for All Samples ($N = 55$)

	repeatability[a]		multivariate relation[b]				direct relation[c]	
phenolic compound	grape	wine	LV[d]	r[e]	RMSEC[f]	RMSECV[g]	r[e]	RMSECV[g]
total phenols (0.01 abs)	4%	1%	2	0.910	68	75 (11%)	0.880	86 (13%)
anth-spec (mg ME/kg)	4%	1%	5	0.932	48	61 (12%)	0.941	67 (13%)
MP (abs)	9%	4%	6	0.896	0.29	0.39 (18%)	0.897	0.45 (20%)
SPP (abs)	9%	1%	5	0.916	0.07	0.09 (13%)	0.801	0.15 (22%)
LPP (abs)	21%	10%	1	0.798	0.08	0.08 (30%)	0.384	0.13 (46%)
tannins (mg CE/kg)	6%	3%	3	0.754	189	205 (24%)	0.653	244 (28%)
PP (abs)	13%	3%	5	0.910	0.12	0.14 (15%)	0.755	0.25 (26%)
gallic acid (mg/kg)	9%	2%	2	0.671	6.8	7.2 (31%)	0.608	7.7 (33%)
(+)-catechin (mg/kg)	4%	4%	8	0.912	11	15 (16%)	0.954	14 (15%)
(−)-epicatechin (mg/kg)	5%	3%	5	0.888	12	13 (17%)	0.948	14 (18%)
hydroxycin. (mg CFAE/kg)	18%	5%	6	0.230	4.5	6.2 (52%)	0.735	6.3 (53%)
flavonols (mg RUE/kg)	3%	5%	7	0.518	19	23 (27%)	0.757	25 (29%)
anth-HPLC (mg ME/kg)	5%	2%	6	0.911	40	53 (13%)	0.934	54 (14%)

[a] The repeatability of from triplicate determinations of three samples (in % of the mean) for both grape and wine. [b] Multivariate relation was evaluated from all 13 phenolic compounds of grapes using PLS model with full cross validation. [c] The direct relation between grape and wine was evaluated using a one factor PLS model with full cross validation. [d] LV is the number of latent variables used for the PLS model. [e] The r value is the correlation coefficient between the predicted and measured color attribute. [f] RMSEC is the root-mean-square error of calibration. [g] RMSECV is the cross validated root-mean-square error of prediction, with the % of the mean given in the brackets.

the study of Romero-Cascales et al. it was demonstrated that seed tannins correlated very well ($r = 0.90$) with wine tannins (*13*), however the increased number of samples in our study seemed to scatter the expected relationship between grape and wine tannins. Wine anthocyanins (by HPLC) were highly correlated with grape anthocyanins ($r = 0.94$), but also with grape total phenols ($r = 0.83$), flavonols ($r = 0.82$), SPP ($r = 0.81$), and polymeric pigments ($r = 0.75$) (**Table 5**). Interestingly, wine polymeric pigments (PP, SPP, and LPP) were all slightly better correlated with grape anthocyanins and total phenols, than grape polymeric pigments, which is in good accordance with the central role of anthocyanins in the formation of polymeric pigments (*3, 4*). Despite the low levels of gallic acid determined in grapes, a good correlation from grape gallic acid to wine (+)-catechin ($r = 0.80$) and (−)-epicatechin ($r = 0.90$) was found. In contrast, the correlation between grape and wine contents of gallic acid was lower ($r = 0.64$). This result may be a consequence of the release of gallic acid from different hydrolysis reactions during winemaking.

Because many wine phenolics correlated well with more than one group of phenolics in the grapes (and vice versa), multivariate PLS models using the complete phenolic profiles of grapes to model the levels of individual wine phenolic compounds were developed and compared with models of the direct relationship from grape to wine for each individual phenolic compound (**Table 6**). In general, the RMSECV values of the multivariate models were only slightly smaller than the RMSECV values for the direct relation between the individual grape and wine phenolics. Apparently, the biggest improvement using multivariate models was obtained for the polymeric pigments (SPP, LLP, and PP), with RMSECV values about 40% lower than for the direct linear relations. The observed minor improvements using multivariate models could be because only small evolutions in the phenolic composition of the wines had occurred at the moment of analysis. The repeatability estimates (**Table 6**) of especially the grape determinations (describing the combined sampling, extraction, and measurements errors) in many cases amounted to a considerable proportion of the model errors (RMSECV in %). The highest proportions were found for MP, SPP, LPP, PP, gallic acid, and hydroxycinnamates.

To exclude any potential variation caused by varietal differences between the grape cultivars, the direct and multivariate relations between grape and wine phenolics were analyzed for only the 27 Merlot samples (**Table 7**). For all phenolic compounds, except gallic acid, the RMSECV values of the direct relation between grape and wine phenolics improved (i.e., both the absolute and the relative percent values of the RMSECV data were lower) when only the Merlot samples were studied, as compared to the analyses done on all the grape samples (cf. **Table 6** with **Table 7**). For the Merlot grapes, the RMSECV values of the multivariate relation between grape and wine phenolics as compared to the direct models were slightly

Table 7. Direct and Multivariate Relation between Grape and Wine Phenolics for Merlot Samples ($N = 27$)

	repeatability[a]		multivariate relation[b]				direct relation[c]	
phenolic compound	grape	wine	LV[d]	r[e]	RMSEC[f]	RMSECV[g]	r[e]	RMSECV[g]
total phenols (0.01 abs)	5%	2%	2	0.857	41	50 (7%)	0.834	54 (7%)
anth-spec (mg ME/kg)	3%	2%	4	0.903	31	41 (8%)	0.920	46 (9%)
MP (abs)	4%	5%	1	0.886	0.20	0.24 (11%)	0.906	0.23 (10%)
SPP (abs)	10%	2%	4	0.782	0.06	0.08 (11%)	0.617	0.13 (18%)
LPP (abs)	7%	10%	1	0.434	0.07	0.08 (27%)	−0.135	0.11 (35%)
tannins (mg CE/kg)	10%	4%	1	0.076	153	170 (15%)	0.641	130 (12%)
PP (abs)	4%	4%	1	0.756	0.13	0.15 (15%)	0.405	0.22 (22%)
gallic acid (mg/kg)	15%	3%	2	0.228	8.4	9.2 (32%)	0.155	9.3 (32%)
(+)-catechin (mg/kg)	7%	2%	2	0.675	16	18 (14%)	0.845	13 (10%)
(−)-epicatechin (mg/kg)	5%	3%	4	0.761	10	13 (11%)	0.865	12 (11%)
hydroxycin. (mg CFAE/kg)	11%	14%	2	0.149	3.6	4.2 (41%)	0.521	3.5 (34%)
flavonols (mg RUE/kg)	7%	10%	2	0.695	22	25 (27%)	0.891	16 (17%)
anth-HPLC (mg ME/kg)	8%	5%	3	0.897	30	38 (10%)	0.888	44 (12%)

[a] The repeatability of from triplicate determinations of one Merlot sample (in % of the mean) for both grape and wine. [b] Multivariate relation was evaluated from all 13 phenolic compounds of grapes using PLS model with full cross validation. [c] The direct relation between grape and wine was evaluated using a one factor PLS model with full cross validation. [d] LV is the number of latent variables used for the PLS model. [e] The r value is the correlation coefficient between the predicted and measured color attribute. [f] RMSEC is the root-mean-square error of calibration. [g] RMSECV is the cross validated root-mean-square error of prediction, with the % of the mean given in the brackets.

Table 8. Relation between Grape Sugar Content (° Brix) and Individual Wine Phenols (Both Total Levels and Ratios) and Modeling of Wine Phenols from Both Grape Phenol and Sugar Levels

	relation between °Brix and wine phenols[a]		relation between °Brix and phenol ratios (wine/grape)[b]		modeling of wine phenols (Merlot) from grape phenols and °Brix[c]		
phenolic cmpound	$r_{\text{all samples}}$	r_{Merlot}	$r_{\text{all samples}}$	r_{Merlot}	LV	RMSECV[d]	r[e]
total phenols (0.01 abs)	0.28	0.24	0.62	0.49	1	44	0.89
anth-spec (mg ME/kg)	−0.08	0.37	0.65	0.55	3	40	0.94
MP (abs)	−0.10	0.38	0.59	0.29	3	0.23	0.90
SPP (abs)	0.06	0.52	0.37	−0.07	1	0.12	0.64
LPP (abs)	0.09	0.48	0.08	0.20	2	0.09	0.30
tannins (mg CE/kg)	0.55	0.16	0.42	0.41	1	123	0.69
PP (abs)	0.07	0.56	0.23	0.22	1	0.18	0.61
gallic acid (mg/kg)	0.35	−0.38	−0.32	−0.34	2	9.5	0.21
(+)-catechin (mg/kg)	0.52	−0.28	0.21	0.12	3	13	0.85
(−)-epicatechin (mg/kg)	0.54	−0.05	0.49	0.07	3	12	0.87
hydroxycin. (mg CFAE/kg)	−0.26	0.12	0.04	0.12	1	3.5	0.52
flavonols (mg RUE/kg)	0.22	0.22	0.36	0.38	1	15	0.91
anth-HPLC (mg ME/kg)	−0.12	0.24	0.50	0.20	3	42	0.90

[a] Direct relation between grape sugar content and total level of wine phenols. [b] Direct relation between grape sugar content and the ratio between wine and grape phenols (**Table 4**). [c] The levels of individual wine phenols was modeled from three variables: the level of the grape phenolic compound, the grape sugar content, and the product between grape sugar and phenol content using PLS with full cross validation and up to three latent variables (LV). [d] RMSECV is the cross validated root-mean-square error of prediction, with the % of the mean given in the brackets. [e] The r value is the correlation coefficient between the predicted and measured levels of the individual wine phenol.

improved for total phenols, anthocyanins, and the various polymeric pigments, but the relation deteriorated somewhat for tannins, flavonols, and (+)-catechin (**Table 7**). These cases of poorer multivariate models — as compared to the direct models — of the relation between grape and wine phenolics may be related to the halving of the number of samples, when analyzing only the Merlot samples.

The evaluation of any eventual impact of the variation in grape sugar content on the extraction of phenolics during fermentation (due to the increased ethanol levels) showed that there was only a weak relation between the grape sugar levels and the total levels of the individual wine phenols (**Table 8**). The correlation coefficients between grape sugar and wine phenolics were also not consistent for all samples as compared to only Merlot samples. This could be a result of cultivar differences skewing the relations between the sugar contents and the phenolic levels. A more consistent relation was found between the grape sugar content and the phenolic ratios (wine/grape), indicating that the grape sugar content slightly impacted the extraction kinetics of the phenols (**Table 8**). To test if the grape sugar could improve the prediction of the levels of wine phenolics, a PLS model was developed from the grape sugar content, the levels of the individual phenolic compound, and the interaction term between these two for only the Merlot samples (**Table 8**). In most cases it was only possible to slightly improve the prediction of wine phenols, as compared to the direct relation between grape and wine phenols (**Table 7**). This indicated that wine phenols primarily correlated with the levels of phenols in the grapes and only to a lesser extent with sugar levels.

Prediction of Wine Color Attributes from Phenolic Profiles. Wine color attributes for all samples were determined after pH normalization (pH = 3.6), allowing comparison of the color attributes without interference from the potential influence of pH on the equilibria between the differently colored forms of anthocyanins. Good correlation to total wine color (i.e., the color after adjustment of pH to 3.6) was found for both wine anthocyanins ($r = 0.986$) and grape anthocyanins ($r = 0.961$), which clearly showed the importance of anthocyanins for the color intensity of young wines. It has been shown that grape anthocyanins can be used for predictive purposes for wine color (*11*). However, molecular associations between pigments and

Table 9. Prediction of Color Attributes of pH Normalized Wines from the Phenolic Profiles of Grapes (see **Table** 2) by PLS Regression

color attribute	mean (±relSD)[a]	Rep[b]	LV[c]	r[d]	RMSEC[e]	RMSECV[f]	RPD[g]
total wine color	11.4 (±49%)	1%	5	0.958	1.15	1.60 (14%)	3.5
wine color due to copigmentation	4.0 (±61%)	3%	5	0.962	0.49	0.66 (16%)	3.7
wine color due to polymeric pigments	1.7 (±36%)	1%	4	0.932	0.18	0.22 (13%)	2.7
wine color due to anthocyanins	5.7 (±47%)	1%	5	0.943	0.61	0.87 (15%)	3.0
tonality	0.47 (±8%)	1%	7	0.713	0.02	0.03 (6%)	1.4
color intensity	1.64 (±47%)	1%	5	0.957	0.16	0.23 (14%)	3.4

[a] Mean values (±relative SD) for the 55 samples. [b] Rep is the estimated repeatability from triplicate determinations of three samples (in % of the mean). [c] LV is the number of latent variables used for the PLS model. [d] The *r* value is the correlation coefficient between the predicted and measured color attribute. [e] RMSEC is the root-mean-square error of calibration. [f] RMSECV is the cross validated root-mean-square error of prediction, with the % of the mean given in the brackets. [g] RPD is the residual predictive deviation calculated as SD/SECV.

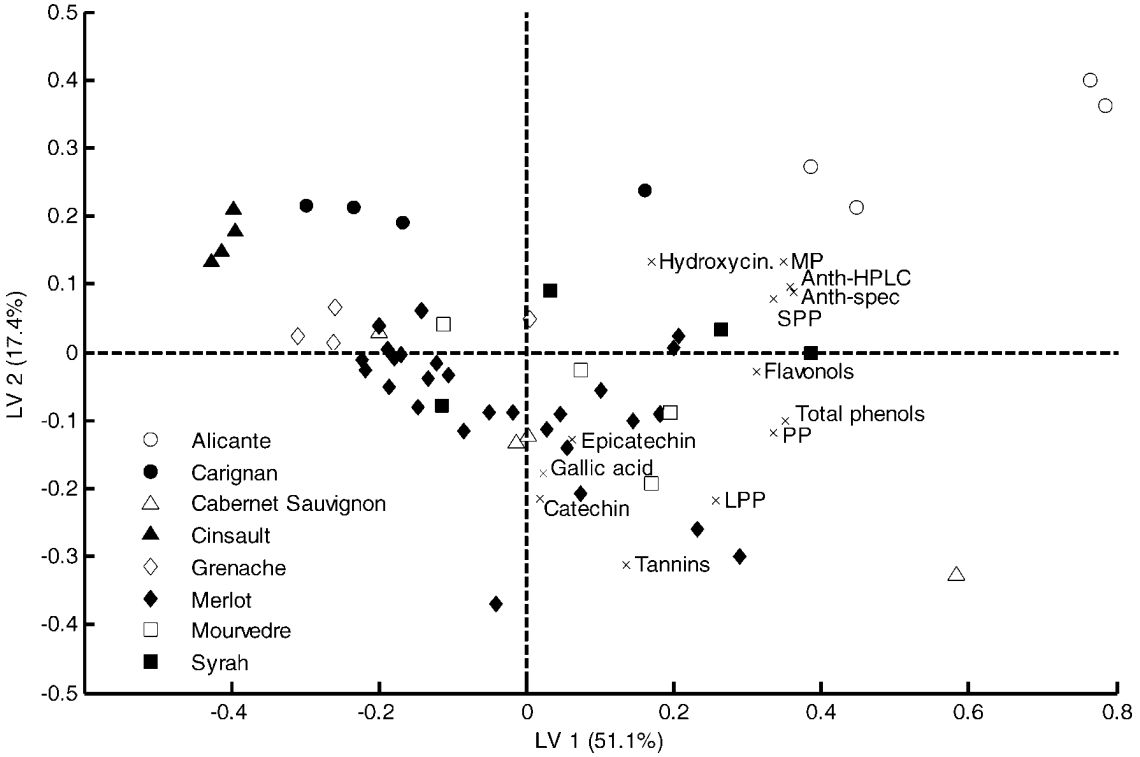

Figure 4. Biplot of the scores and loadings from the partial least-squares regression of total wine color from the detailed phenolic composition of grapes (as in **Table 2**).

noncolored compounds (copigmentation cofactors) are known to strongly increase the red wine color intensity, in some cases up to 50% (*14*). Whereas the color of grapes and young wines is dominated by anthocyanins, these compounds are not very stable, and their color impact moreover varies with pH. As the wine ages, anthocyanins both degrade and condense with other compounds, in particular tannins, producing more stable pigments (*3*). Therefore, red wine color depends not only on the actual concentration of the anthocyanins and the pH, but also on the levels polymeric pigments and copigmentation cofactors, in particular other phenolic compounds.

Color analysis with Boulton's assay (*20*) made it possible to quantify the average percentage of color due to anthocyanins (51%), polymeric pigments (16%), and copigmentation (34%) in the wines. Realizing that the wine color is not only a product of the concentration of anthocyanins, we investigated if using detailed phenolic profiles of grapes would improve the prediction of total wine color and allow prediction of other wine color attributes. The residual predictive deviation (RPD) is a good tool for evaluating model performance, and in general, calibrations with RPD values greater than three are considered to be very good for prediction purposes (*27*). Total wine color (RPD = 3.5), color due to copigmentation (RPD = 3.7), color due to anthocyanins (RPD = 3.0), and color intensity (RPD = 3.4) were predicted very well from the phenolic profiles of the grapes (**Table 9**). Probably due to a lower variation between the samples (relative SD = 36%), color due to polymeric pigments was slightly more difficult to predict (RPD = 2.7). Likewise, color tonality was poorly predicted (RPD = 1.4); this might be ascribed to a very low variation between samples (relative SD = 8%). The repeatability estimates for all the color attributes (**Table 9**) were much lower than the RMSECV percentages and were likely to have a smaller effect on the model errors than the repeatability of the grape measurements (**Table 6**).

The biplot for the PLS regression model for total wine color (**Figure 4**) was very similar (with an opposite sign on the second latent variable) to the PCA of the phenolic composition of grapes (**Figure 2**) and showed that the first latent variable, which was the most important for wine color, once again was associated with the variation on anthocyanins, polymeric pigments, total phenols, and flavonols.

The predicted total wine color correlated well with the measured total wine color (i.e., the color measured after normalization of the pH of the wines to 3.6) ($r = 0.958$; **Figure 5**). This confirmed that prediction of the total wine color from the phenolic composition of grapes could be accomplished by

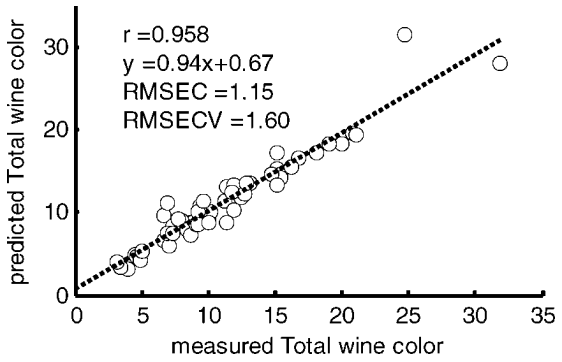

Figure 5. Relationship between the measured and predicted values (by cross validation) of the PLS regression model of total wine color.

multivariate regression, at least when wines were normalized to the same pH, thereby avoiding confoundings from the influence of pH on the color response by anthocyanins. The data obtained is a first step in providing a prediction of wine color from grape phenolic profile analysis. However, the direct relation between grape anthocyanins and total wine color (r = 0.961) was just as good as the relation between the measured and predicted total wine color found in the multivariate model (r = 0.958; **Figure 5**). Hence, determination of only grape anthocyanins is sufficient to obtain a satisfactory prediction of total wine color in very young wines. The relation between the grape anthocyanins and total wine color found in this study was in good accordance with the reported correlation of $R^2 = 0.82$ by Iland (*11*).

In the present study, the wines were produced in a laboratory scale setup, and the evolution of the phenolic profiles — and the putative alterations in wine color attributes — during maturation, aging, and prolonged storage, were not examined. The average total color in the present study (11.4 absorbance units, **Table 9**) was slightly higher than the average reported total color of young commercially produced Cabernet Sauvignon wines as measured by the same method (8.2 absorbance units) (*20*). The color value obtained was also higher than the reported average total color (approximately 4.5 absorbance units) of commercial wines — also measured by the same method — covering a wide range of cultivars (*28*). The higher color values in the present study were probably a result of the fact that only freshly fermented wines were examined. For practical and comparative (precision) purposes, frozen grape material was used as the starting material in the present work. The extraction of phenolic compounds from frozen grapes might therefore have been higher than for fresh grapes (*9*). Also, wine phenolics and color attributes do change during extended maturation and storage of wines. It is worth noting, however, that the color values obtained were nevertheless of the same order of magnitude as those reported previously for commercial wines. The data obtained signify that it is possible to predict the color quality of fresh wines from grape measurements and they thus provide an important starting point for further identification and prediction of wine quality parameters from grape measurements. The integration of the current data with data obtained in large-scale commercial wine making will be an important next step in the prediction of wine color from grapes.

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Paper IV

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Identification of Spectral Regions for the Quantification of Red Wine Tannins with Fourier Transform Mid-Infrared Spectroscopy

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Accomplishment of fast tannin measurements is receiving increased interest as tannins are important for the mouthfeel and color properties of red wines. Fourier transform mid-infrared spectroscopy allows fast measurement of different wine components, but quantification of tannins is difficult due to interferences from spectral responses of other wine components. Four different variable selection tools were investigated for the identification of the most important spectral regions which would allow quantification of tannins from the spectra using partial least-squares regression. The study included the development of a new variable selection tool, iterative backward elimination of changeable size intervals PLS. The spectral regions identified by the different variable selection methods were not identical, but all included two regions (1485−1425 and 1060−995 cm^{-1}), which therefore were concluded to be particularly important for tannin quantification. The spectral regions identified from the variable selection methods were used to develop calibration models. All four variable selection methods identified regions that allowed an improved quantitative prediction of tannins (RMSEP = 69−79 mg of CE/L; $r = 0.93-0.94$) as compared to a calibration model developed using all variables (RMSEP = 115 mg of CE/L; $r = 0.87$). Only minor differences in the performance of the variable selection methods were observed.

KEYWORDS: FT-MIR spectroscopy; tannins; red wine; variable selection; partial least-squares regression

INTRODUCTION

Tannins are the most abundant group of phenolic compounds typically found in red wines (*1*), and the tannins play an important role in the mouthfeel properties and color stability of red wines (*2–4*). According to their chemical structure, tannins in wines are commonly classified as either condensed tannins or hydrolyzable tannins (**Figure 1**). Condensed tannins originate primarily from the skins and seeds of grapes and are oligomers or polymers of flavan-3-ol subunits (termed catechins), whereas hydrolyzable tannins mainly originate from oak (and thus occur in wines that have been aged in oak barrels) and are gallic acid and/or ellagic acid esters of glucose (*5, 6*).

Tannins have the ability to precipitate with proteins present in saliva. This interaction is presumed to be responsible for the astringent sensation of red wines (*2*). The ability to precipitate with proteins has been used for the quantitative analysis of tannins with bovine serum albumin (BSA) (*7, 8*). Tannin concentrations measured by protein precipitation have been found to correlate particularly well with the perceived astringency of red wines (*4*), and tannin analysis by protein precipitation has been recommended within winery settings (*9*). As reviewed elsewhere, several other principles for tannin analysis in wines have been reported, for example, precipitation by methyl cellulose, HPLC, and various colorimetric assays (*6, 10*). These types of methods are all slow, and the time requirement for accomplishing these tannin analyses currently represents a major obstacle for the implementation of such tannin analysis in the array of routine wine quality control measurements at wineries. Due to the increasingly recognized importance of tannins and hence tannin measurement in relation to red wine quality, a significant need thus exists for more rapid analytical techniques for quantification of tannins.

Employment of Fourier transform mid-infrared (FT-MIR) spectroscopy has recently emerged as a possible solution for rapid measurement of wine tannins (*11, 12*). FT-MIR has already found use in the industry for the analysis of several other important components in wine, including ethanol, organic acids, and sugars (*13, 14*). Interference between the characteristic absorption bands of major wine components and tannins poses a problem for direct quantification of tannins in wines by infrared spectroscopy. This problem has been overcome by sample purification using solid phase extraction (*11*), but again this strategy is not feasible for

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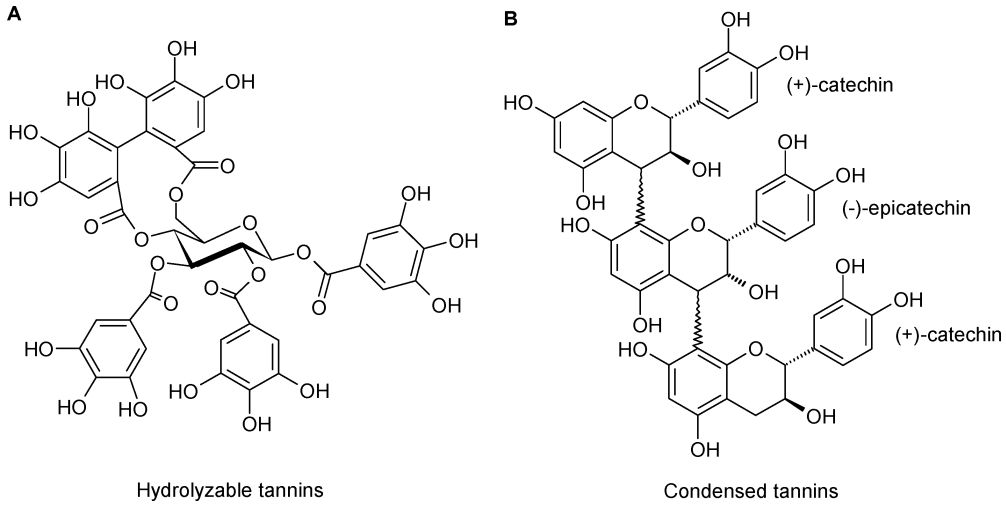

Figure 1. Examples of chemical structures of the two different classes of tannins: hydrolyzable tannins (**A**) and condensed tannins (**B**).

accomplishing rapid tannin analyses in industrial wine production. An alternative way is to identify the characteristic spectral regions of tannins, which do not suffer from this interference, and in turn use this identification to develop calibration models that then allow the rapid quantitative assessment of tannins by FT-MIR. A number of tools to identify important spectral regions for improving partial least-squares (PLS) calibrations are available and include synergy interval PLS (*15*), backward interval PLS (*16*), and genetic algorithm PLS (*17*). In brief, some of the main features of the methods are the following: synergy interval PLS finds the combination of up to four spectral intervals, which leads to the best PLS model; backward interval PLS eliminates the most noninformative regions of the spectra iteratively; and, finally, the genetic algorithm PLS finds the best combinations of spectral intervals using an evolutionary approach. One particular drawback of these present methods is that they all require predefined interval sizes, which may lead to identification of spectral intervals covering both noninformative and informative regions.

This study was undertaken to assess and compare different variable selection methods for the identification of important spectral regions for the quantification of red wine tannins by FT-MIR spectroscopy and the method of PLS regression. Furthermore, we wanted to evaluate the applicability a new variable selection method involving an iterative backward elimination of changeable size intervals.

MATERIALS AND METHODS

Materials. Chemicals for tannin analysis, including BSA (fraction V powder), tartaric acid, potassium tartrate, sodium dodecyl sulfate (SDS), triethanolamine (TEA), ferric chloride hexahydrate, and (+)-catechin hydrate, were all of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO). Commercial tannin extracts from grapes (tannin grape) and oak wood (Tannivin Superb) were purchased from Erbslöh Geisenheim AG (Geisenheim, Germany). One hundred and twenty-eight commercial red wines were purchased from local shops in Denmark. The wines were selected to represent a wide range of different vintages (11 vintages ranging from 1996 to 2006), grape varieties (covering at least 30 different varieties), and production countries (16 different countries).

Mid-Infrared Spectra. Spectra in the mid-infrared range were measured by Fourier transform interferometry on a Winescan Auto spectrometer (FOSS, Hillerød, Denmark) equipped with a liquid flow system and a 37 μm calcium fluoride cuvette, thermostated at 40 °C. Transmission infrared spectra of 1060 data points in the range between 5012 and 926 cm^{-1} of all wines were measured in triplicate. The noninformative and noisy parts of the full spectra were removed to give the "good range" region of 265 data points in the following regions: 2969−2699, 1812−1716, and 1577−933 cm^{-1} (**Figure 2**).

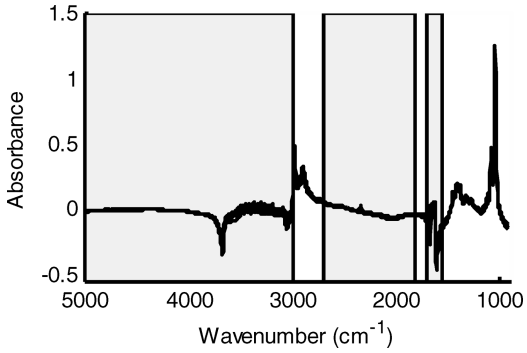

Figure 2. FT-MIR spectra of the 128 commercial wines (5012−926 cm^{-1}). The noninformative and noisy parts (specified by the gray rectangles) of the spectra are discarded to retain only the "good range" spectral regions.

Tannin Analysis. Tannin concentrations of all wines were measured in duplicate using a slightly modified method of Harbertson et al. (*7*). Briefly, the method relies on tannins being precipitated with BSA, redissolved and measured by a color reaction with ferric chloride. Prior to analysis, wines were diluted in a model wine solution of 12% v/v ethanol containing 5 g/L of tartaric acid, which had been adjusted to a pH value of 3.3 with NaOH. Modifications to the original method were as follows: The precipitation step was conducted for 30 min instead of 15 min, the centrifugation speed for forming the tannin−protein pellet was increased from 13500*g* to 14000*g*, and finally the SDS/TEA buffer volume for redissolving the tannin−protein pellet was increased from 0.875 to 1.5 mL to allow background measurement (A^{BG}) on a 1 mL sample, which was then reacted with 0.125 mL of iron chloride (11.4 mM $FeCl_3$ in 11.4 mM aqueous HCl), and the absorbance was measured after 10 min (A^{FeCl3}). Dilutions of the sample in the model wine solutions were carried out to give a tannin response (calculated as $1.125A^{FeCl3}$ minus A^{BG}) between 0.3 and 0.75, which was defined as the valid range of the assay (*18*). Tannins were reported in milligrams of catechin equivalents (CE) per liter from a linear standard curve of the color reaction between catechin and ferric chloride [absorbance = 0.006258 × (concentration of catechin in mg/L); $r = 0.9997$].

Spiking Experiments. Separate solutions with 2 g/L oak tannin, (+)-catechin, and grape tannin respectively dissolved in 20% v/v aqueous ethanol were prepared, and the FT-MIR spectra of the solutions were measured. The spectral characteristics of the three products were determined as the difference between the FT-MIR absorbance spectra of the solutions and the FT-MIR absorbance spectra of the aqueous ethanol solution. A red wine (Cabernet

Sauvignon, Chile, 2005) was spiked with different levels (0, 0.1, 0.2, 0.5, 1.0, 1.5, and 2.0 g/L) of grape tannin and analyzed by FT-MIR spectroscopy. For each spiking level, the dose−response signal was evaluated as the difference between the FT-MIR absorbance spectrum of the spiked wine sample and the unspiked wine. The wine was analyzed to have a tannin level of 298 mg of CE/L, and the grape tannin powder contained 355 mg of CE/g of tannin powder.

Model Development. Multivariate calibration models were developed in MATLAB R14 (MathWorks, Natick, MA) using the PLS toolbox 4.02 (Eigenvector Research, Natick, MA). The infrared absorbance spectra were mean centered, and calibration models for measurement of the determined components were developed with PLS regression using cross-validation in nine segments. The triplicate spectra were included in the models to make it possible for the model to compensate for replicate variations between the spectra. The optimal number of latent variables in each model was determined from the minimum root-mean-square error of cross validation (RMSECV), allowing a maximum of 10 latent variables. The first 81 wines were used for developing the calibration models both for the "good range" region (265 data points), the fingerprint region from 1577 to 933 cm^{-1} (168 variables), the expected main region for phenolics from 1157 to 1577 cm^{-1} (110 variables), and for spectral regions of the reduced spectrum identified by different variable selection methods described below. The ability of the developed calibration models to predict the tannin concentration in wines was evaluated using an independent validation set of 47 wines to calculate the correlation coefficient between the measured and predicted values and the root-mean-square error of prediction (RMSEP). The wines used for validation were analyzed at a different point of time from the calibration wines to ensure independence of the validation set.

Variable Selection Methods. Four different variable selection methods were used to develop calibration models from the calibration set (81 wines) with segmented cross-validation in 9 segments, allowing up to 10 latent variables. The prediction performance of the calibration models was evaluated from the external validation set (47 wines). The following variable selection methods were evaluated: backward interval PLS (*16*) (bi-PLS; using 17 intervals and up to 10 latent variables), synergy interval PLS (*15*) (si-PLS; using 17 intervals, 4 regions, and up to 10 latent variables), genetic algorithm PLS (*17*) (GA-PLS; using a window size of 15 and up to 10 latent variables), and iterative backward elimination of changeable size intervals PLS (IBECSI-PLS, as described below) and compared with models developed using manually selected spectral intervals. The predictive performances of the models were compared pairwise from a F test of the RMSEP values: $F(n_1,n_2) = \mathrm{RMSEP}_1^2/\mathrm{RMSEP}_2^2$, on a $p < 0.05$ level (*19*).

Iterative Backward Elimination of Changeable Size Intervals PLS. A new variable selection method, "iterative backward elimination of changeable size intervals PLS" (IBECSI-PLS), was developed using the PLS toolbox 4.02 (Eigenvector Research). The IBECSI-PLS method works by an iterative elimination of intervals of changeable sizes from the spectra, by minimizing the RMSECV of the PLS model (**Figure 3**). The intervals were found by the following routine: The spectra were divided into a number of equally sized intervals (here 20 intervals), and PLS regression models with each of the intervals left out were calculated. The center point of the interval, which gave the lowest RMSECV when left out, was set as the starting point for the region to be eliminated (step 1 in **Figure 3**). The region to be eliminated was then stepwise expanded one data point at a time in the direction (left or right) causing the lowest RMSECV(step 2 in **Figure 3**) and repeated until a local minimum of RMSECV was found and the region was eliminated (step 3 in **Figure 3**). Steps 1−3 were repeated for the reduced spectra, and the routine was repeated until an optimal region could be identified (step 4 in **Figure 3**)—as discussed under Results and Discussion.

RESULTS AND DISCUSSION

Tannins in Wines. The concentration of tannins in the 128 commercial wines ranged from 92 to 1060 mg of CE/L (**Table 1**) and covered the most typical values of tannin concentrations in red wines reported by others using the same analytical method (*4, 7, 11, 12, 20, 21*). However, in some cases tannin levels have been reported as high as 1655 mg of CE/L in commercial wines (*12*). For the development of the calibration models for the quantification of tannins by FT-MIR spectroscopy using the protein precipitation data as reference, samples were split into a calibration set (81 wines) and a validation set (47 wines) with similar standard deviations and comparable ranges of the tannin levels (**Table 1**).

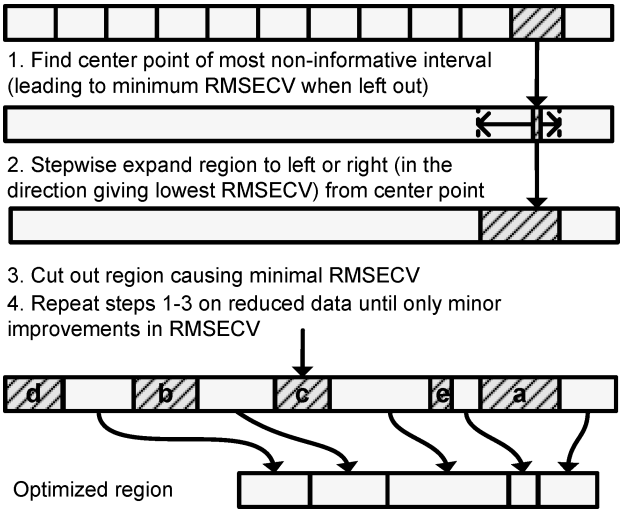

Figure 3. Overview of IBECSI-PLS for variable selection.

Table 1. Descriptive Statistics of the Red Wine Samples Used for Calibration and Validation

		tannin concentration[a]		
sample	N[b]	range	mean	SD
all samples	128	92–1060	456	181
calibration	81	112–1060	472	180
validation	47	92–830	429	181

[a] Tannin concentration in mg of CE/L. [b] Number of samples.

Spectral Features of Tannins. The spectral response of a commercial grape tannin product at different levels in the FT-MIR spectrum of a selected red wine (Cabernet Sauvignon, 2005) was investigated (**Figure 4**). Although the absorbance values of the tannin signals were very low compared to the wine spectra (**Figure 2**), there was a systematic spectral dose−response effect of the added grape tannins, which was particularly evident in the regions 2969−2699 and 1577−1060 cm^{-1} (**Figure 4**). On the other hand, small or no distinct grape tannin signals were evident in the regions from 1812 to 1716 cm^{-1} and from 1060 and 933 cm^{-1}. The occurrences of the small negative absorbances observed in some regions were primarily ascribed to small drifts in the FT-MIR spectra with time. The most prominent signals for the grape tannins were: two major peaks at 1520 and 1445 cm^{-1} in the typical region of aromatic ring stretches (*22*) (**Table 2**), a peak at 1285 cm^{-1}, corresponding to the C−O stretch of the pyran derived part of flavonoid based tannins (*5*), and several peaks between 1400 and 1050 cm^{-1}, in the overlapping regions of OH stretch and deformations of phenols and CH deformations in aromatic compounds (*22*).

The relatively small spectral response from tannins in the wine spectra, combined with the known absorptions in the same spectral region as tannins of major wine components, such as ethanol and organic acids (*23*), complicates the use of infrared spectroscopy for the quantification of tannins in wine. Variable

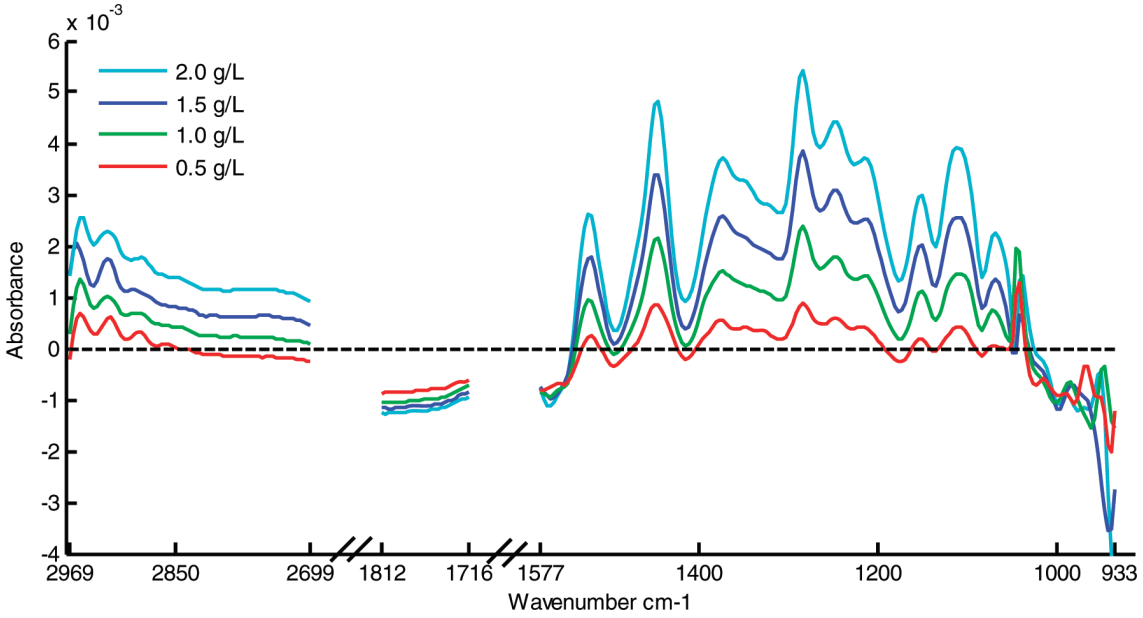

Figure 4. Spectral response of grape tannins to the spectra of a red wine at different concentrations (0.5, 1.0, 1.5, and 2.0 g/L) in the "good range" regions.

Table 2. Known Mid-Infrared Bands for Tannins in the Informative Regions (2996−2699, 1812−1716, and 1577−933 cm^{-1}) of the Spectrum (*5, 11, 22*)

functional group	group frequencies (cm^{-1})
aromatic overtones and combinations	2000–1700
C═O stretch of esters	1750–1740
C═C stretch of aromatic rings	1650–1430
C—O—H deformation of phenols	1390–1310
C—OH stretch of phenols	1340–1160
C—O stretch of flavonoid pyran ring	1285
C—H in-plane deformation of aromatic compounds	1270–1000

selection provides a way to remove interfering or noninformative regions of the infrared spectra, by which the models may be improved and in turn improve the accuracy of tannin measurement.

Iterative Backward Elimination of Changeable Size Intervals PLS. A new variable selection, IBECSI-PLS, which iteratively removes continuous regions from the spectra, was developed. As opposed to many other variable selection methods, the interval size of the eliminated region in IBECSI-PLS is found by a stepwise expansion of the region to be removed, which could be useful when informative and interfering spectral features are close. The IBECSI-PLS method was applied to the tannin data to remove the (differently sized) interfering or noninformative regions from the spectra. Due to the risk of overfitting the data by too extensive an elimination of variables, it is important to eliminate variables only while it gives a considerable decline in the model error and to validate the performance of the final model with independent samples. The optimal number of iterations was manually set to 11, because only minor declines in the model error were observed in the further iterations (**Figure 5B**). Additionally, only a few variables were removed per iteration in the further iterations (**Figure 5A**), also indicating that little further improvement was possible. The validation of the model performance with independent samples is discussed further below.

Spectral Regions for Tannin Quantification. The four variable selection methods for finding the best regions in the "good range" of the IR spectra were evaluated to see if the calibration models could be improved. The spectral regions identified either manually or by the variable selection methods are illustrated in **Figure 6** and compared with the spectral characteristics of red wine, oak tannin, (+)-catechin, and grape tannin. The spectral characteristics of oak tannin, grape tannin, and (+)-catechin were similar to the reported spectral characteristics of hydrolyzable tannins, grape tannin, and (+)-catechin (*5*). Although the regions identified by the four variable selection methods were not identical, two regions were selected by all four methods: the region from 1060 to 995 cm^{-1}, which was dominated by high absorption of the OH stretch in ethanol, and the region between 1485 and 1425 cm^{-1}, at which grape tannin gave a distinct absorption peak (**Figure 6**). Furthermore, all variable selection methods included wavelengths in the region from 2969 to 2699 cm^{-1} (**Figure 6**). The selected regions were, however, not the same for the different methods and, due to the lack of distinct peaks, thereby likely functioned as reference points in the spectra. Both bi-PLS and IBECSI-PLS retained wavelengths around 1750 cm^{-1}, with IBECSI-PLS retaining a much narrower region than bi-PLS. Oak tannins had a spectral response matching the C═O stretches of the ester group typically found in hydrolyzable tannins (**Figure 1**) and wavelengths around 1200 cm^{-1}, which matched some of the region of C-OH stretches of phenols (**Table 2**). Others have reported that the wavelength around 1285 cm^{-1} corresponds to the C—O stretch of the flavonoid pyran ring structure and may be used to distinguish condensed and hydrolyzable tannins (*5*). However, none of the variable selection methods retained the region around 1285 cm^{-1} for the quantitative analysis of tannins from the FT-MIR spectra of red wines. The elimination of this region for tannin quantification may be a consequence of the overlapping peak from (+)-catechin and the missing peak from oak tannins at 1285 cm^{-1} (**Figure 6**).

Measurement of Red Wine Tannins with Mid-Infrared Spectroscopy. The performances of the PLS models for measurement of wine tannins were evaluated from the prediction errors (RMSEP) and correlation coefficients (r_{val}) between the actual and predicted tannin concentrations of independent validation samples (**Table 3**). The results showed that the PLS

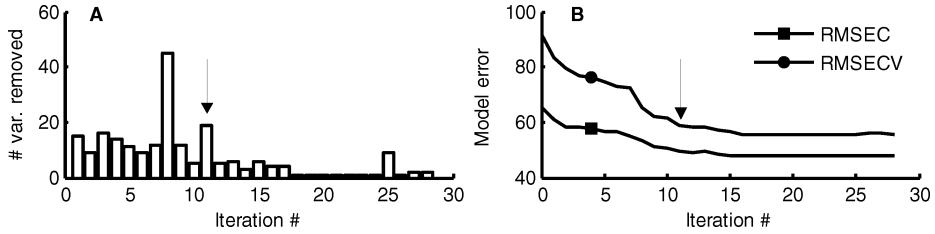

Figure 5. Variable selection by IBECSI-PLS results in iterative elimination of variables from the data (**A**), which attempts to minimize the model error (**B**). The arrow indicates optimal number of iterations.

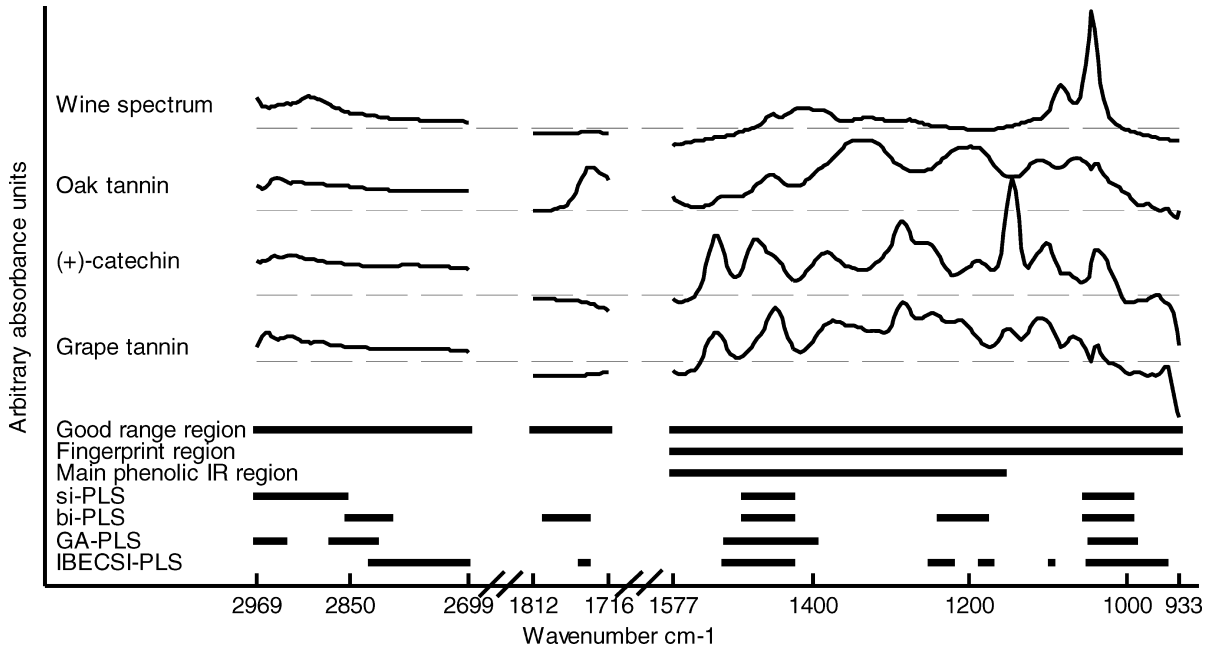

Figure 6. Identified spectral regions for tannin quantification obtained by different variable selection procedures in relation to the IR spectrum of a wine sample (scaled down 100 times) and the IR signals of oak tannin, (+)-catechin, and commercial grape tannin.

Table 3. Overview of Calibration and Validation Results for Quantification of Tannins in Red Wines from PLS Models Using the Spectral Regions Identified from Variable Selection Methods

selected variables	no. of variables	LV[a]	RMSEC[b]	RMSECV[b]	RMSEP[b,c]	r_{val}[d]
good range region	265	10	65	92	115 c	0.87
fingerprint region	168	10	69	91	92 bc	0.91
main phenolic region	110	10	54	75	88 b	0.90
si-PLS region	62	10	53	65	77 ab	0.93
bi-PLS region	78	10	53	65	69 a	0.94
GA-PLS region	70	9	55	69	79 ab	0.93
IBECSI-PLS region	97	10	49	59	75 ab	0.94

[a] Number of latent variables. [b] Root mean square error of calibration, cross-validation, and prediction, respectively, in mg of CE/L. [c] The same letters indicate no significant ($p < 0.05$) differences between the predictive abilities of the models. [d] Correlation coefficient between the measured and predicted tannin levels.

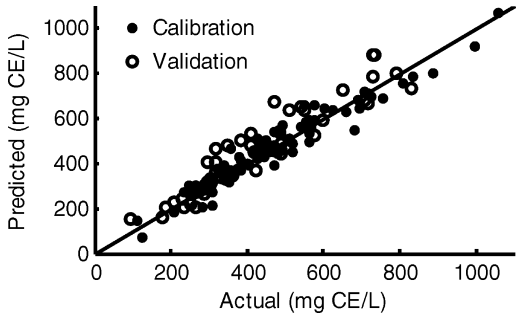

Figure 7. Measurement of red wine tannins (in mg of CE/L) by FT-MIR spectroscopy using the spectral region identified by IBECSI-PLS variable selection method.

model using the main phenolic region was significantly better than the model using all variables in the "good range", decreasing the RMSEP values from 115 to 88 and increasing the correlation coefficients from 0.87 to 0.91 (**Table 3**). Improvements in the RMSEP values to between 69 and 79 mg of CE/L and the correlation coefficients to ~ 0.94 were obtained for the four variable selection methods. Although all models using the four variable selection methods were significantly better than using the "good range" region, only the bi-PLS model was statistically better than the model of the main phenolic region (**Table 3**). The differences in the RMSEP values between the four variable selection methods were relatively small and were not found to be statistically different from each other (**Table 3**). Recently it was shown that little or no improvement in the measurement of tannins by mid-infrared spectroscopy was obtained by variable selection, when the majority of the interfering substances were removed using solid phase extraction and evaporation (*11*). The considerable improvements by variable selection found in this study were ascribed to the presence of major interferences from other wine components in the infrared spectra.

Figure 7 shows the correlation between the actual and predicted levels for the model developed from the region identified by IBECSI-PLS. The repeatability of tannin levels (in percentage of the mean value) predicted from the triplicate

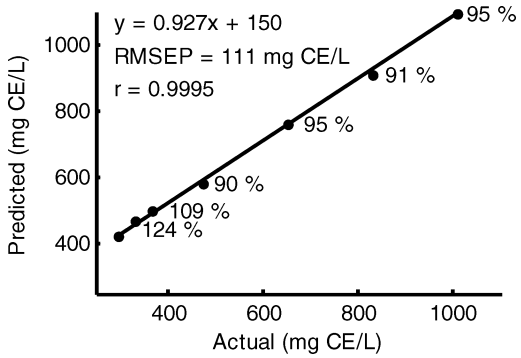

Figure 8. Prediction of tannin content by FT-MIR spectroscopy of a red wine (having 298 mg of CE/L tannin) spiked with 0, 0.1, 0.2, 0.5, 1.0, 1.5, and 2.0 g/L grape tannin corresponding to 0, 36, 71, 178, 355, 533, and 710 mg of CE/L. The recovered tannin amount (as a percentage of the added amount) is given next to the data points.

spectra of the validation samples was 3.5%. This was considerably higher than the repeatability of the tannin analysis reference method of 0.95% determined from duplicate measurements, but still acceptable. The prediction ability of the developed model (RMSEP = 75 mg of CE/L; r = 0.94; **Table 3**) was not as good as the model reported by Fernandez et al. (RMSEP = 51 mg of CE/L; r = 0.96), which, however, includes only a single grape cultivar and requires extensive sample purification (*11*). The prediction ability was similar to the reported values of Versari et al. (RMSECV = 63 mg of CE/L; r = 0.99), who also used FT-MIR spectroscopy, but their method was developed using a high number of latent variables for only 20 wines without any independent validation of the model (*12*). Skogerson et al. have recently shown that tannins can be measured by ultraviolet−visible (UV−vis) spectroscopy (RMSEP = 66 mg of CE/L; r = 0.93) in young Australian wines and fermenting juices (*20*). A similar accuracy (RMSEP = 75 mg of CE/L; r = 0.94) was found in our study with commercial wines covering a wide range of vintages, production countries, and grape cultivars using FT-MIR spectroscopy. The performance of the developed model was further tested for its ability to predict the tannin levels in a red wine spiked with different levels of grape tannin (**Figure 8**). Although the tannin content of the unspiked wine was predicted to be considerably higher (422 mg of CE/L) than the actual level (298 mg of CE/L), the spiked tannin levels gave a good linear response (r > 0.99) and acceptable recoveries for tannin levels higher than ~71 mg of CE/L.

The present study demonstrated that particularly important spectral regions could be identified almost equally well by the four variable selection methods: si-PLS, bi-PLS, GA-PLS, and IBECSI-PLS. The identified regions could be used to develop calibration models, which allowed the measurement of tannins in wines by FT-MIR spectroscopy. The results obtained demonstrate that FT-MIR spectroscopy (coupled with a proper calibration model) is a good option for the rapid quantification of tannins in red wines.

ABBREVIATIONS USED

BSA, bovine serum albumin; CE, (+)-catechin equivalents; LV, latent variables; PLS, partial least-squares; RMSEC, root-mean-square error of calibration; RMSECV, root-mean-square error of cross-validation; RMSEP, root-mean-square error of prediction; SD, standard deviation; SDS, sodium dodecyl sulfate; TEA, triethanolamine; UV−vis, ultraviolet−visible.

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SUPPLEMENTARY INFORMATION

Sample #	Data set	Vintage	Wine name	Country	Primary grape variety	Tannins (mg CE/L)
1	Calibration	2000	Castillo de Moral	Spain	Tempranillo	565
2	Calibration	2005	Drostdy-Hof	South Africa	Pinotage	380
3	Calibration	2003	Primitivo di Manduria	Italy	Zinfandel	352
4	Calibration	2004	Santa Isabel Merlot Reserva	Argentina	Merlot	548
5	Calibration	2003	Barbaresco Morando	Italy	Nebbiolo	999
6	Calibration	2004	Villagio Merlot	Romania	Merlot	520
7	Calibration	2004	Feudo Martinella	Italy	Negroamaro	493
8	Calibration	2001	Veccia Cantina	Italy	Sangiovese	717
9	Calibration	2002	Grande Sommellerie	France	Merlot	384
10	Calibration	2004	San Felice	Italy	Sangiovese	561
11	Calibration	2004	Woodbridge Zinfandel	USA	Zinfandel	578
12	Calibration	2001	Faustino V	Spain	Tempranillo	431
13	Calibration	2005	Undurraga Merlot	Chile	Merlot	403
14	Calibration	2005	Undurraga Merlot Reserva	Chile	Merlot	404
15	Calibration	2001	Barolo San Martino	Italy	Nebbiolo	833
16	Calibration	2005	Beaujolais-Villages	France	Gamay	494
17	Calibration	N/A	Coteaux Du Languedoc	France	N/A	564
18	Calibration	2006	Lindemans Bin 45	Australia	Cabernet Sauvignon	282
19	Calibration	2005	Requevin	Spain	Tempranillo	888
20	Calibration	2005	Undurraga Pinot Noir	Chile	Pinot Noir	265
21	Calibration	2002	Ch. St. Benoit de Ferrand	France	Merlot	512
22	Calibration	2003	Sutter Home Cab. Sauv.	USA	Cabernet Sauvignon	354
23	Calibration	N/A	Jeanne d'Arc	France	N/A	348
24	Calibration	2004	Les Marches de Caïx	France	Malbec	756
25	Calibration	2005	Lindemans Bin 50	Australia	Shiraz	232
26	Calibration	2005	Santa Isabel Malbec	Argentina	Malbec	309
27	Calibration	2004	Inglewood Cab. Sauv.	South Africa	Cabernet Sauvignon	307
28	Calibration	2006	Trivento	Argentina	Merlot	276
29	Calibration	2002	Barolo Riva Leone	Italy	Nebbiolo	489
30	Calibration	2005	Albarutta Zitto Zitto	Italy	Sagrantino	543
31	Calibration	2003	Roaix Cotes du Rhône Villages	France	Grenache	809
32	Calibration	2004	Dom. De la Curniére	France	Grenache	578
33	Calibration	2003	Amarone Farina	Italy	Corvina	691

Sample #	Data set	Vintage	Wine name	Country	Primary grape variety	Tannins (mg CE/L)
34	Calibration	2001	Ciró Rosso Classico	Italy	Gaglioppo	1060
35	Calibration	2005	Montepulciano d'Abruzzo Aldi	Italy	Montepulciano	381
36	Calibration	1996	Val Conde Gran Reserva	Spain	Tempranillo	472
37	Calibration	2000	Dão Terras da Feira	Portugal	Touriga Nacional	444
38	Calibration	2004	Turner Estate Ruby Cab.	USA	Ruby Cabernet	249
39	Calibration	2001	Vega Calinda	Spain	Tempranillo	465
40	Calibration	2001	Ch. Gragnos	France	Syrah	373
41	Calibration	2005	Il Tasso Chianti	Italy	Sangiovese	511
42	Calibration	2005	Argento Reserva Bonarda	Argentina	Bonarda	707
43	Calibration	2004	Lenz Moser Blaufränkich	Austria	Blaufränkich	207
44	Calibration	2003	Chateau d'Arsac	France	Cabernet Sauvignon	564
45	Calibration	2004	La Consulta Syrah	Argentina	Syrah	438
46	Calibration	2005	Acacia Hill Shiraz	Australia	Shiraz	112
47	Calibration	2005	Manfredi Barbera d'Asti	Italy	Barbera	444
48	Calibration	2001	Botter Merlot Riserva	Italy	Merlot	682
49	Calibration	2003	Warburn Estate "1164"	Australia	Cabernet Sauvignon	659
50	Calibration	2005	Simonsvlei Lifestyle Shiraz	South Africa	Shiraz	428
51	Calibration	2005	Santa Helena Siglo de Oro	Chile	Carmenére	295
52	Calibration	2005	Cotes-du-Rhone Brugsen	France	Grenache	470
53	Calibration	2004	Ch. Graves de Marchesseau	France	Merlot	471
54	Calibration	1999	Ramon Bilbao Reserva	Spain	Tempranillo	482
55	Calibration	2002	Seventh Moon	USA	Cabernet Sauvignon	338
56	Calibration	2003	Montepulciano d'Abruzzo Novacorte	Italy	Montepulciano	433
57	Calibration	2004	Cuvée Prestige Skouras	Greece	Aghiorgitiko	364
58	Calibration	2004	Regnie Mommesin	France	Gamay	470
59	Calibration	2001	Amarone Pagus Bisano	Italy	Corvina	725
60	Calibration	2003	Cantore Nero d'Avola	Italy	Nero d'Avola	448
61	Calibration	2004	Ch. Les Vignes Des Genets	France	Merlot	693
62	Calibration	2005	Flor de Montgó	Spain	Monastrell	601
63	Calibration	2004	Bourgogne de Légende	France	Gamay	268
64	Calibration	2003	Domaine Astruc Pinot Noir	France	Pinot Noir	358
65	Calibration	2002	Groval Bairrada Reserva	Portugal	Baga	358
66	Calibration	2004	Bourgogne Pinot Noir Reine Pedaque	France	Pinot Noir	322
67	Calibration	1997	Castillo de Ezpeleta	Spain	Tempranillo	349
68	Calibration	2005	Valmaduro Cab. Sauv.	Chile	Cabernet Sauvignon	309

Sample #	Data set	Vintage	Wine name	Country	Primary grape variety	Tannins (mg CE/L)
69	Calibration	2004	Don Mena Cab.Sauv. Reserva	Chile	Cabernet Sauvignon	418
70	Calibration	2003	Gray Fox	USA	Cabernet Sauvignon	519
71	Calibration	2005	Hardy's Varietal Range Shiraz	Australia	Shiraz	124
72	Calibration	2004	Quintus Valpolicella	Italy	Corvina	403
73	Calibration	2005	Savanha Merlot	South Africa	Merlot	487
74	Calibration	2005	Savanha Shiraz/Cab.Sauv./Pinotage	South Africa	SCP	434
75	Calibration	2005	Savanha Shiraz	South Africa	Shiraz	288
76	Calibration	2005	Bourgogne Pinot Noir Sebastien Roux	France	Pinot Noir	352
77	Calibration	2004	Santa Ana Reserve Malbec	Argentina	Malbec	555
78	Calibration	2005	Argento Reserva Malbec	Argentina	Malbec	429
79	Calibration	2003	Story Ridge Zinfandel	USA	Zinfandel	246
80	Calibration	2003	Principe de Viana	Spain	Cabernet Sauvignon	555
81	Calibration	2002	Quinta del Rio	Spain	Tempranillo	624
82	Validation	2004	Castillo de Farnatella	Italy	Sangiovese	485
83	Validation	N/A	Vox Populi Merlot	Romania	N/A	551
84	Validation	2001	L.A. Cetto Cabernet Sauvignon	Mexico	Cabernet Sauvignon	471
85	Validation	2005	Clos Gebrat	Spain	Grenache	543
86	Validation	2001	Sizzano	Italy	Nebbiolo	486
87	Validation	2005	Cycles Gladiator Pinot Noir	USA	Pinot Noir	237
88	Validation	2002	Nugan Alcira Cab. Sauv.	Australia	Cabernet Sauvignon	423
89	Validation	2002	Kressman Monopole	France	Merlot	408
90	Validation	2004	Rio Sol	Brazil	N/A	734
91	Validation	2002	Primitivo Zonin	Italy	Zinfandel	511
92	Validation	2004	Nugan Manuka Grove Durif	Australia	Petite Sirah	600
93	Validation	2004	EagleHawk Shiraz	Australia	Shiraz	177
94	Validation	2004	Finca Flichman Reserva Malbec	Argentina	Malbec	295
95	Validation	2004	Zalse Pinotage/Shiraz	South Africa	Pinotage	319
96	Validation	2005	Cono Sur Carmenere	Chile	Carmenére	488
97	Validation	2000	Villachica Crianza	Spain	Tempranillo	457
98	Validation	2003	Inca Merlot/Bonarda	Argentina	Merlot	318
99	Validation	2004	Fleur des Bois Julienas	France	Gamay	265
100	Validation	2001	Barbaresco Riserva Ricossa	Italy	Nebbiolo	791
101	Validation	2002	Rowan's Ridge Cab/Merlot	South Africa	Cabernet Sauvignon	331
102	Validation	2004	Ch. Martignan	France	Cabernet Sauvignon	460
103	Validation	2003	Barbera d'Alba Dessilani	Italy	Barbera	707

Sample #	Data set	Vintage	Wine name	Country	Primary grape variety	Tannins (mg CE/L)
104	Validation	2002	Angove's CR Cab. Sauv.	Australia	Cabernet Sauvignon	264
105	Validation	2005	La Natura Salento	Italy	Zinfandel	349
106	Validation	2004	Santa Rita Merlot	Chile	Merlot	361
107	Validation	2003	Primavera Bairrada Reserva	Portugal	Baga	830
108	Validation	2002	Bourgogne Grand Ordinaire	France	Pinot Noir	185
109	Validation	2005	California Spring Cab. Sauv.	USA	Cabernet Sauvignon	288
110	Validation	2001	Copertino Rosso	Italy	Negroamaro	206
111	Validation	2004	Laurence Feraud Cotes du Rhone	France	Grenache	417
112	Validation	2005	Bush Wine	South Africa	Cabernet Sauvignon	408
113	Validation	2004	Rasteau Cloitre Noir	France	Syrah	578
114	Validation	2005	Cono Sur Cab. Sauv.	Chile	Cabernet Sauvignon	565
115	Validation	2000	Pepi Merlot	USA	Merlot	295
116	Validation	2003	Prieure Ksara	Lebanon	Cinsault	728
117	Validation	2003	Reserve du Couvent	Lebanon	Syrah	730
118	Validation	2004	Gallo Sierra Valley Zinfandel	USA	Zinfandel	319
119	Validation	2005	Custoza Corvina Veronese	Italy	Corvina	295
120	Validation	2002	Cuvée du President	Algeria	N/A	92
121	Validation	2003	Nederburg Shiraz	South Africa	Shiraz	253
122	Validation	1998	Chateau de Caïx	France	Malbec	715
123	Validation	2005	Vila Bona Terra Pinot Noir	Romania	Pinot Noir	339
124	Validation	2000	Ch. Vieux l'Estage	France	Merlot	342
125	Validation	1997	Gran Civet Hill Crianza	Spain	Tempranillo	382
126	Validation	2001	Domaine Eole	France	Carignan	304
127	Validation	1999	Castillo del Baron Gran Reserva	Spain	Tempranillo	229
128	Validation	2005	Trapiche Cab. Sauv. Astica	Argentina	Cabernet Sauvignon	650